School of Pharmacy

Isolation, Identification and Characterisation of Antibacterial Compounds from *Carissa lanceolata* R.Br. Root

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Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Signature: …….

Date: ……...

This Work is Dedicated to My Beloved Parents

"All things which are old should not necessarily be true; all things which are new should not necessarily be without fault. To the wise men both of them should be acceptable only if they stand to the test. The unwise, however, gets swayed by the current of other's opinion"

- Kalidasa's Malakavikagniputhra Opera

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Abbreviations

DCM	-	Dichloromethane
MeOH	-	Methanol
EtOAc	-	Ethyl acetate
EtOH	-	Ethanol
MIC	-	Minimum inhibitory concentration
NMR	-	Nuclear magnetic resonance
^{1}H	-	Proton
^{13}C	-	Carbon 13 isotope
TLC	-	Thin layer chromatography
GC	-	Gas chromatography
MS	-	Mass spectroscopy
GC-MS	-	Gas chromatography with mass selective detector
IR	-	Infra red spectroscopy
UV	-	Ultra violet
SPME	-	Solid Phase Micro-Extraction
s	-	Singlet ^{1}H NMR signal
d	-	Doublet ^{1}H NMR signal
t	-	Triplet ^{1}H NMR signal
q	-	Quadruplet ^{1}H NMR signal
ppm	-	Parts per million

Abstract

Carissa lanceolata (conkerberry) is a perennial woody shrub used in traditional medicine by indigenous communities in Western Australia, the Northern Territory and Queensland for various medical conditions such as toothache, respiratory infections and the cleaning of sores, which all strongly indicate an antibacterial activity. A literature review revealed that the wood of this plant possesses significant antibacterial activity, which was found to be related to the presence of eudesmane type sesquiterpenes. *C. edulis* and *C. carandus* are frequently used in other traditional systems of medicine in different parts of the world, and thus have also been investigated for bioactive compounds and pharmacological properties. Some of these were found to be in line with the main findings of this work.

Carissa lanceolata root was shown to exhibit significant antibacterial activity against both Gram negative and Gram positive organisms. A micro-broth dilution assay was performed on 96-well plates using resazurin as an indicator for microbial growth of *Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis* and *Staphylococcus aureus.* Bioassays carried out in this work showed that crude extracts of root bark and wood, particularly their polar constituents were more active against the four strains of bacteria tested. Chemical investigation of the root bark revealed that it contains a volatile oil, which was isolated by steam distillation as well as solid phase micro extraction. It was found to consist of a single compound, which was identified as 2'-hydroxy acetophenone. The identity of this compound was confirmed by GC/MS and ^{1}H NMR spectroscopy. Furthermore, the eudesmane-type sesquiterpene, carissone, was isolated from the root bark DCM and root wood hexane extracts. Its chemical identity was confirmed by IR, ^{1}H and ^{13}C NMR spectroscopy. The lignan, carinol, on the other hand, was isolated from the moderately polar fractions of the root wood MeOH extract. The obtained IR and ^{1}H NMR data as well as R_f values all correspond to the literature. Two other yet unidentified compounds were isolated, but further studies into their chemistry and antibacterial activity were not possible in this current study. The antibacterial activity of the isolated compounds was considerable, with 2'-hydroxy acetophenone exhibiting the strongest effect, followed by carinol and then carissone.

1 Background

The conkerberry, *Carissa lanceolata* R.Br (Apocynaceae) is commonly used in many Indigenous Australian communities across Northern Australia for the treatment of a variety of conditions such as chest pain, toothache, colds and flu[1, 2]. Elsewhere, other species of the family are also frequently used in traditional systems of medicine in different parts of the world. Of those, particularly *C. edulis* and *C. carandas* have been investigated for bioactive compounds and pharmacological properties (section 1.3 and 1.4). *C. lanceolata*, however, has received considerably less scientific attention despite its important role in traditional native Australian medicine. To date, only the anti-microbial properties of the plant's wood have been investigated in detail[3].

Guided by previous published work on members of the genus and the family (Apocynaceae), a bioassay-guided study using a micro broth dilution technique was carried out in this research project to extract and isolate anti-microbial compounds from root material of *C. lanceolata*. The structures of the isolated pure compounds were elucidated by a variety of instrumental techniques, most importantly Nuclear Magnetic Resonance (NMR) and infra-red (IR) spectroscopy as well as gas chromatography with mass selective detection (GC/MS).

In the following, an overview of the botanical and chemical characteristics as well as the traditional medicinal uses and pharmacological properties of various *Carissa* spp. are provided with particular emphasis on the two most researched members of the genus, *C. edulis* and *C. carandus*, as well as *C. lanceolata* as the topic of this thesis. This background section is followed by a description of the materials and methods used in this project before its findings are presented and discussed. The concluding chapter summaries the major findings and outlines a future research agenda.

1.1 The Genus *Carissa*

The genus *Carissa* belongs to the order Gentianales (Apocynaceae)[2]. Generally, the Apocynaceae family, including the genus *Carissa* contains numerous plants that have been used in traditional medicine and / or have yielded pharmacologically active compounds[4] (Table 1). Their botanical, chemical, medicinal and ethnopharmacological properties is discussed in the following sections in greater detail.

Table 1: Ethnopharmacological use of *Carissa* species

Species	Part used	Ethnopharmacological uses
C. lanceolata	Root	Tooth ache, chest pain, cleaning sores[1, 2]
	Stem	Rheumatism[1]
	Leaves	Cold and flu, as a mosquito repellent[1, 2]
C. carandas	Root	Stomachic, antidiarrheal, anthelmintic[5-7]
	Stem	Strengthens tendons[8]
	Leaves	Fevers, earache, soreness and syphilitic pain[5-7]
	Fruit	Skin infections[9] edible[5,10]
C. edulis	Root	Headache, chest complaints, rheumatism, gonorrhoea, syphilis, rabies, gastric ulcer, glandular inflammation[11-14]
	Fruit	Edible[11]
C. spinarum	Root	Purgative, cleaning wounds in animals[5]
	Fruit	Edible[5]
C. congesta	Fruit	Edible[5]
C. grandiflora (*C. macrocarpa*)	Fruit	Edible[7]
C. opeca	Leaves	Tanning agent[5]
C. bisponosa	Fruit	Edible[5]

1.1.1 Habitat

Thirty to thirty five *Carissa* species are found in Africa, Madagascar, the Arabian peninsula, the Indian subcontinent, New Guinea, New Caledonia and Australia. Of the four species occurring in Australia, three are considered endemic[16] (Table 2).

Table 2: Distribution of Carissa species[5]

Species	Habitat
C. lanceolata	Australia
C. ovata	Australia, New Caledonia
C. laxiflora	Australia
C. scabara	Australia
C. carandus	Indian subcontinent, South East Asia
C. congesta	Indian subcontinent, South East Asia
C. gagentica	Indian subcontinent
C. grandiflora (C. macrocarpa)	Indian subcontinent
C. opeca	Indian subcontinent
C. spinarum	Indian subcontinent (desert areas)
C. bisponosa	Indian subcontinent (desert areas)
C. paucinervia	Indian subcontinent (Himalayan regions)
C. edulis	Africa

1.1.2 Morphology

According to Foster[16] members of the *Carssia* genus are evergreen perennial woody shrubs, scramblers or trees, producing a white latex. The stem has opposite, simple or forked spines. Leaves are petiolate and opposite, glabrous or with basifixed indumentum, collectors being absent. The inflorescence is terminal or extra-axillary, generally a pedunculate of umbelliform. Flowers are scented and pedicellate with the calyx lacking colleters. The corolla is of salverform with a cylindrical tube, usually somewhat bulging around the anthers. The flowers are normally glabrous or with internal and external indumentum, stamens are included, inserted near the top of the tube and not adherent to the style-head, while the anthers are linear to linear-lanceolate. The style-head is subglobose and bifid at the apex. The fruit is a globose

to obloid-ovoid, syncarpus fleshy berry. It contains one to four seeds, which are flattened, globose, unwinged and ecomose[16].

1.1.3 Ethnopharmacology, Chemistry and Pharmacology

The medicinal uses of *C. lanceolata*, the subject of this project, and also *C. carandas,* and *C. edulis*, which both have provided useful points of reference for this work, are discussed in detail in the ensuing sections. Medicinally of lesser importance, and therefore receiving considerably less attention in academic literature, are *C. bispinosa, C. macrocarpa, C. congesta* and *C. spinarium*. The latter produces edible fruits[18], its roots are traditionally used for their purgative properties as well as to treat worm infested wounds in animals[6]. Leaf extracts of *C. opeca*, on the other hand, are used as a tanning agent[6]. Limited chemical and pharmacological studies have not yet been undertaken with those particular *Carissa* species, but lignans, sesquiterpenes of the eudesmane type and several cardiac glycosides have been isolated from them[8]. Moreover, in line with its traditional uses, leaves of *C. opeca* were found to contain tannins whereas the roots of *C. spinarum* were reported to contain caffeic acid while its leaves produce urosolic acid and naringin[5]. Carissone and the D-glucoside of β-sitosterol were isolated from the roots of *C. congesta*[18] and a new germacrane derivative, carenone, was isolated from the stems of *C. spinarum*. The leaves of the latter exhibited significant antibacterial activity against Gram negative organisms[17] . Chloroform extracts of the same displayed strong antioxidant properties[19]. The alcoholic extracts of aerial parts of *C. congesta*, on the other hand, were found to have CNS depressant activity in mice[21]. The cardiotonic activity of *C. spinarum* was four to six times greater than that of *C. carandus*, which will be discussed in more detail in Section 1.3[22].

1.2 *Carissa lanceolata* R.Br.

1.2.1 Habitat

Carissa lanceolata R.Br. or conkerberry (conkleberry), is a poisonous plant found in the open woodlands and stony locations of tropical areas in Western Australia, the Northern Territory and Queensland[24]. Although occurring mostly in humid areas, it has also been found to a limited extent in some desert areas of central Australia[16] (Figure 1).

Figure 1: Geographical distribution of *C. lanceolata*

(Source: Adapted from Forster PI. Flora of Australia: CSIRO Australia; 1996)

1.2.2 Morphology

Carissa lanceolata is a divaricately branched spiny shrub, which grows up to 2 m high, usually glabrous or minutely hairy. Its spines are spreading and straight, 5-7mm long. The leaves are of short petiolate, narrowly elliptic, usually glabrous, and abruptly acuminate. The cymes are often on short branchlets, axillary, where bracts are linear-subulate. Calyx lobes are subulate and acute, up to 2mm long, but often unequal in size. The corolla is white in colour, glabrous from the outside, but sparsely hairy inside. Its lobes are narrowly oblong and subacute. The style is

globular with two appendages. Young berries are red, turning black at maturity and are ovoid to globular in shape[15] (Figure 2).

Figure 2: Arial part of *C. lanceolata*

(Source: www.alicesprings.nt.gov.au)

1.2.3 Ethnopharmacology

The roots, leaves, bark and twigs of *C. lanceolata* have traditionally been used by indigenous communities of Western Australia (WA), Queensland and the Northern Territory (NT) of Australia. It has been used for the treatment of various medical conditions, including toothache, respiratory infections and the cleansing of sores, the causes of which could be bacterial. Some indigenous communities in the Kimberly area of WA burn leaves as a mosquito repellent and squeeze the wood of young plants to obtain a sap, which is rubbed on the skin for rheumatism. Decoction of leaves and twigs are also used by the Amanbidji community (Timber Creek Region, NT) for the treatment of colds and flu-like symptoms. The Lajamanu (CMB Lajamanu, NT) and Yuelamu (Mt. Allen Station, NT) communities prepare a wash from the young roots, which is then rubbed into the skin to treat chest pain. The same decoction is also used by the Borroloola community (Borroloola, NT) to treat toothache[24].

1.2.4 Chemistry

In a previous study, crude extracts of the roots and stems (Figure 2 and Figure 3) have been reported to show antibacterial activity but no detailed follow up chemical analysis was ever undertaken. Furthermore, antibacterial compounds from the aerial

wood of *C. lanceolata*, isolated in a bioassay-guided study, have been reported by Lindsay *et al.*[3]

With respect to its chemical constituents, thus far, the eudesmane-type sesquiterpene, carrisone, and the cardiac glycoside, odoroside H[25], and have been isolated and identified from the roots of *C. lanceolata*[25]

Figure 3: Root of *C. lanceolata*

(source: School of Pharmacy, Curtin University)

1.3 *Carissa carandus* Linn.

1.3.1 Habitat

Carissa carandas occurs in the drier sandy and rocky soils throughout the Indian subcontinent and South East Asia. It is commonly found in Southcentral India and Northern Sri Lanka[6,7] .

1.3.2 Morphology

Carissa carandus is a small tree or a large shrub, with numerous, divaricate branches and very sharp horizontal spines, which are often branched. Its leaves are simple, opposite, oblong-oval or oblong-lanceolate, subacute at the base, obtuse at the apex, glabrous and thin. The flowers are regular and bisexual in threes. They are shortly stroked in clusters at the end of short, axillary and terminal peduncles, which are bract, small and linear with five fused puberulous sepals. The segments are linear or lanceolate, acute and ciliate, lapping to the right. There are five distinct stamens, inserted in the corolla tube, the ovary is superior to locular. The style is a simple stigma conical and the berries are smooth, ovoid and bluntly pointed, reddish-purple, with four seeds[7] (Figure 4).

Figure 4: Aerial part of *C. carandas*

(Source: www.pharmazie.uni-greifswald.de)

1.3.3 Ethnopharmacology

C. carandas is the best known member of the genus, as it has been used as a traditional medicinal plant over thousands of years in the Ayurvedic system of medicine as it is practiced on the Indian subcontinent[5]. Thus, traditional uses of *C. carandas* are well established. The root is credited with bitter, stomachic, antidiarrhoeal and anthelmintic properties, while its leaves are prescribed in remittent fevers, earache, soreness and syphilitic pain of the mouth[6,7]. A tincture of fruits is used in skin infections[9] and a decoction of wood is employed as a tonic to strengthen the tendons of slim patients[8].

1.3.4 Chemistry

The roots of *C. carandas* have yielded a number of volatile principles, including 2-acetylphenol[26,27]. Pal *et al*. have reported a new lignan, carinol [28], from the root of *C. carandas*, whereas studies carried out by Rastogi *et al.* have led to the isolation of a mixture of sesquiterpenes, namely carissone and carindone, as a novel type of C_{31} terpenoid[29-30]. The leaves were reported to have triterpenoidal constituents[33], as well as tannins[34], and a new isomer of urosolic acid, named carissic acid[35], was also found. Fruits of this plant were demonstrated to contain a mixture of volatile principles[36, 27] including 2-phenyl ethanol, linalool, β-caryophyllene, isoamyl alcohol, benzyl acetate and a novel triterterpenic alcohol, carissol[37]. Enzymatic and mild hydrolysis

of polar glycosides from *C. carandas* yielded oderoside H, digitoxigenin and the sugars D-glucose and D-digitalose[29].

1.3.5 Pharmacology

Pharmacological studies have identified cardiotonic activity in an alcoholic extract of root material and noted a decrease of blood pressure in normal anaesthetised cats[38]. The total crude plant extract produced an increase in free histamine in guinea pig lung and a pronounced decrease in blood pressure is known to be caused by the plant's lipophilic fraction[39]. Isolated carissone derivatives exhibited an antizymotic, antibacterial and atropine like spasmolytic activity[40]. The lipase activity of fruits has also been studied[41], while an aqueous extract of the root demonstrated anthelmintic, spasmolytic, cardiotonic and hypertensive action in a preliminary pharmacological screening undertaken by Zaki *et al.* [26] An antipyretic activity has also been reported for the same extract[42].

1.4 *Carissa edulis* Vahl

1.4.1 Habitat

C. edulis is found throughout the Arabian peninsula and occurs also in warm humid and hot dry areas of tropical Africa, the Transvaal, Botswana, north and northeast Namibia[11].

1.4.2 Morpholgy

Carissa edulis (Figure 5) is a spiny, much branched, small tree, shrub or scrambler with a milky sap, which grows up to 5 m in height. Its bark is grey and smooth, young branchlets are with or without hairs. The spines are simple, straight and usually single. Its leaves are opposite and ovate to ovate-elliptic. They are coriaceous, dark green above and paler green below, glabrous to pubescent. Lateral veins are obscure, the apex is tapering often with a bristle-like tip, while the base is rounded to shallowly lobed, the margins are entire with a 1-4 mm long petiole. The sweetly scented flowers are white tinged with purple, red or pink, up to 1.8 cm long, about 2 cm in diameter, slender and tubular, with the corolla lobes overlapping to the right.

Fruits are ovoid to almost spherical, red-black when young, upon ripening turning purplish black and contain two to four flat seeds[11].

Figure 5: Aerial parts of *C. edulis*

(Source: pharmazie.uni-greifswald.de)

1.4.3 Ethnopharmacology

C. edulis is a well-known African member of the *Carissa* genus commonly used in African folk medicine, especially in the northern and eastern countries of the continent[14]. Traditional applications include treatment of headache, chest complaints, rheumatism, gonorrhoea, syphilis and rabies[12-14]. The plant's roots have been used in Africa for a variety of medicinal purposes. The vapour from a hot aqueous root bark infusion, for example, is inhaled as a treatment for chest congestion and the root is also applied as poultice to relieve toothache[12, 13]. In Ghana, the root bark is mixed with spices and used as an enema for lumbago and other pains, while root scrapings are used for glandular inflammation. Ground up roots are employed as a remedy for venereal diseases, to restore virility, to treat gastric ulcers, cause abortion and as an expectorant[11]. An infusion of roots along with other medicinal plants can be used to treat chest pains and a root decoction is also used for treating malaria[11].

1.4.4 Chemistry

Carissone, cryptomeridiol, β-eudesmol , a sesquiterpene of the eudesmane type with a yet to be identified structure, and a germacrane derivate have all been isolated from the roots of this species[43]. Studies of *Carissa edulis*' lignan fraction by Achenbach *et al.* resulted in the identification of carissanol, (-)-nortrachelogenin and carinol, other lignans found to be present are (+)-lariciresinol , (-)-scoisolariciresinol and

(-)-olivil[44]. The roots also contain 2'-hydroxy acetophenone, vanillin, 4-hydroxy-(3-hydroxypropionyl)-benzene, coniferaldehyde, scopoletin, isofraxidin and catalponol[44,45]. Furthermore, another study has identified the presence of γ-eudesmol[46]. The inositol derivative quebrachitol in the twigs and an alkaloid in the stem and leaves have also been reported[46].

1.4.5 Pharmacology

In pharmacological studies, root wood extracts were found to induce a diuretic effect in rats[48]. Furthermore, an extract of the leaves of *C. edulis* significantly reduced the blood glucose level in streptozotocin diabetic rats, indicating the presence of compounds with hypoglycaemic activity[49]. Aqueous extracts of *C. edulis* have exhibited significant activity against the *Herpes simplex* virus (HSV) *in vitro* and *in vivo* for both wild type and resistant strains of HSV[50].

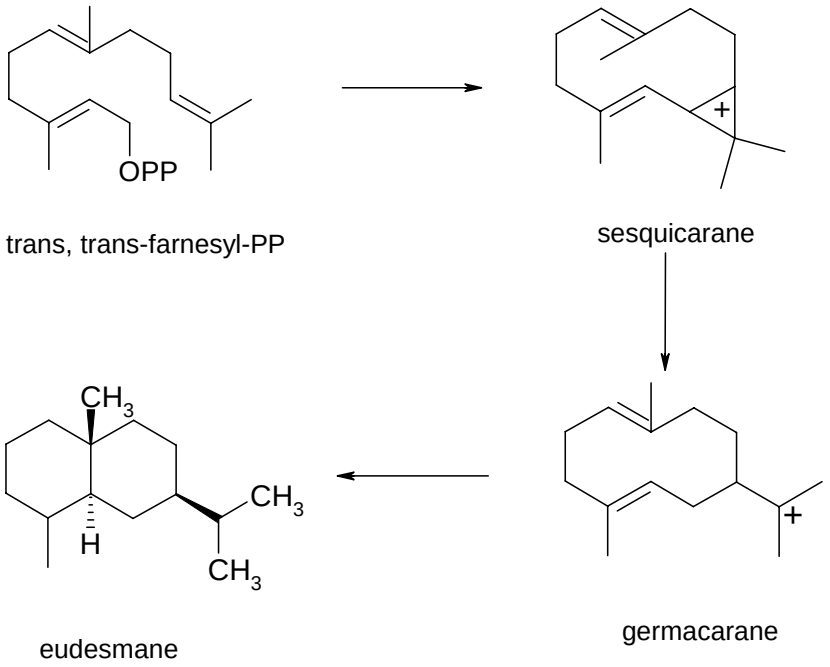
1.5 Reported compounds

In the following section, key compounds isolated from the various *Carissa* species are discussed in more detail with respect to their chemistry.

1.5.1 Sesquiterpenes

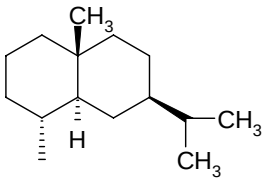
Sesquiterpene are C_{15} compounds biogenetically derived from farnesyl pyrophosphate [51] (Figure 6).

Figure 6: Biogenesis pathway of sesquiterpenes[51]

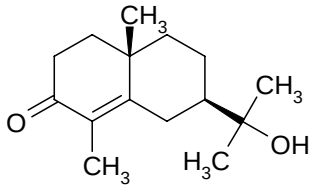


Sesquiterpenes of the eudesmane type (1), such as Carissone (2), are one of the genus' characteristic compounds, but they have also been isolated from genera other than *Carissa*, for instance from *Makania banisteriae*[52], *Allophylus laevigatus*[53], *Persea japonica*[54] and *Eucalyptus macarthuri*[55]. When inoculated with TMV virus, *Nicotina undalata* leaves were found to produce stress compounds including dehydrocarissone (3) together with another 18 sesquiterpenes[56]. In general, sesquiterpenes are known to possess antimicrobial, antineoplastic and anti-inflammatory actions[47]. Eudesmanoids from *Parthenium argentatum* and *P. tomentose* were, for instance, shown to exhibit a significant antifungal effect[57].

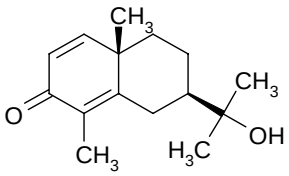
Figure 7: Sesquiterpenes from *Carissa* spp



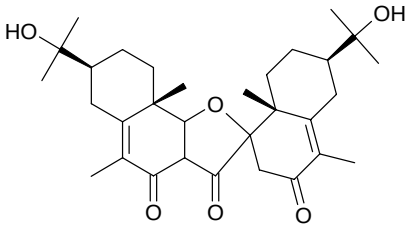
Eudesmane skeleton(1)



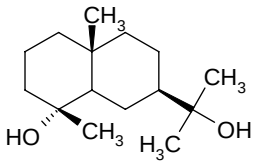
Carissone (2)



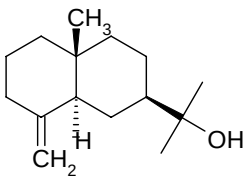
Dehydrocarissone (3)



Carindone (4)



Cryptomeridol (5)



β-Eudesmane (6)

Carissone (1) was obtained by complete synthesis using santonin as a starting material[58] and has also been synthesised from eudesmol for the purpose of establishing its absolute stereochemistry[59]. A series of substituted hydrazones,

carbohydrazones and thiocarbohydrazones have also been synthesised using carissone, α-cyperone, 4,5-dihydrocarissamine and 5-cyanocarissone as starting materials[59].

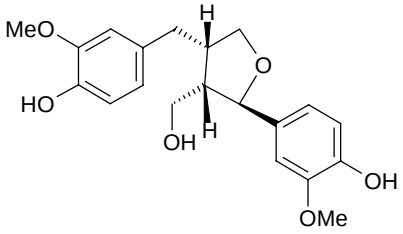
Carindone (4) is a new C_{31} type terpenoid isolated from *C. carandas* and *C. lanceolata*, which displays a significant antimicrobial activity[3].

1.5.2 Lignans

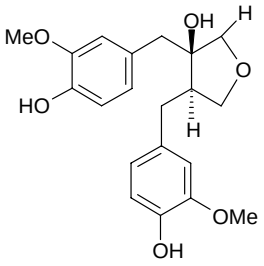
Biogenetically, lignans are derived from the Shikimic acid - Cinnamic acid pathways, followed by a series of enzymatic reactions to form these dimeric molecules[60]. They display antimicrobial and antioxidant activities due to their polyphenolic nature[61].

Lignans reported from *Carissa* species[44] also commonly occurring in trees of both, Gymnospermae and Angispermae [61]. The lignan carinol (7) was first isolated from *C carandas*[28], while carissanol[44] (8) was obtained from *C. edulis*. Other lignans reported from *C. edulis*[44] are secoisolariciresinol (9), nortrachelogenin (10) and olivil (12) as shown in figure 8.

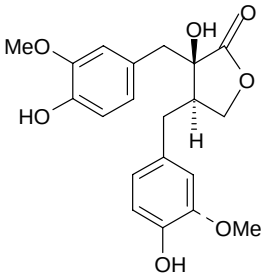
Figure 8: Lignans identified from *Carissa* spp. [44]



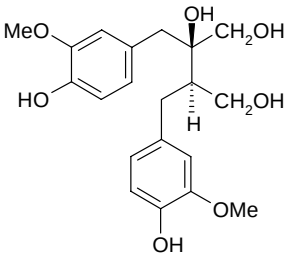
Carinol (7)



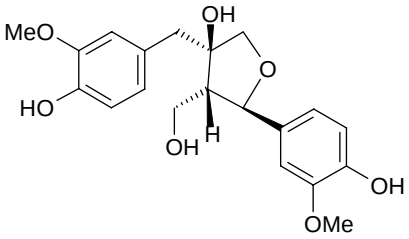
Carsissanol (8)



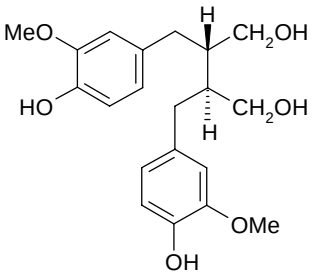
Secoisolariciresinol (9)



(-)-Nortrachelogenin (10)



(+)-Rariciresinol (11)

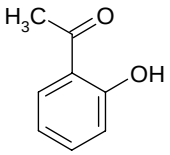


(-)-Olivil (12)

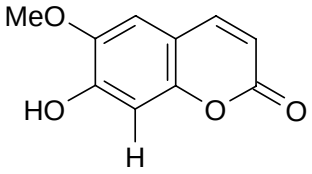
1.5.3 Other isolated compounds

2-Hydroxy acetophenone (13) has been isolated by steam distillation from the fresh root bark of *C. edulis*[45]. Although floral extracts of *C. carandas* were free from 2-hydroxy acetophenone, they contained many other volatile compounds[36] such as vanillin (17), scopoletin (16), coniferaldehyde (15), γ-eudesmol and 4-hydroxy-(3-hydroxypropionyl)benzene (14) which are illustrated in figure 9.

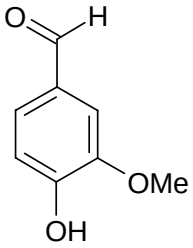
Figure 9: Other compounds isolated from *Carissa* spp.



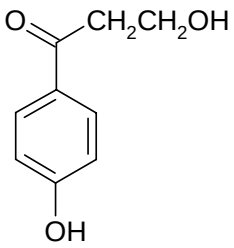
2'-Hydroxy acetophenone (13)



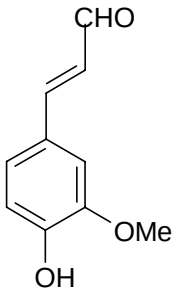
2'-Hydroxy-(3-propnyl)benzene (14)



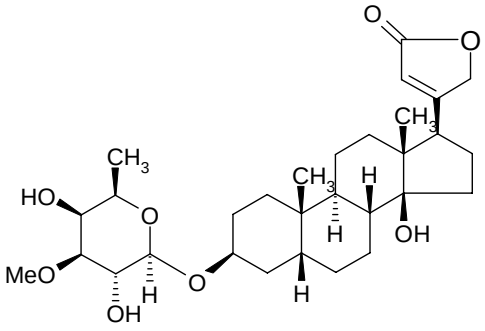
Coniferaldehyde (15)



Scopoletine (16)



Vanillin (17)



Odoroside H (18)

Odoroside H (18), odoroside G, evomonoside and perhaps two other as yet structurally unidentified cardiac glycosides are responsible for the observed cardiotonic activity of *C. carndas* and *C. spinarum* [22]. Moreover, stem bark and leaves of *C. spinarum* have been reported to contain sitosterol, lupeol and urosolic acid[17]. Odoroside H has also been isolated and reported from *C. lanceolata* roots by Mohr *et al*[25].

1.6 Antibacterial activity

Roots of *Carissa* species are often used in traditional systems of medicine due to their antibacterial activities (Table 1). *C. lanceolata* root, for instance, is used by many Aboriginal communities in central and northern Australia to treat toothache and to clean sores. The root of *C. edulis* is used as an antiseptic in Ghana, while in East and Northern African folk medicine it plays an important role in treating syphilis and gonorrheae. *C. carandas* root is prescribed in the Ayurvedic system of medicine as an antidiarrheal and anthelmintic. Indian farmers use a decoction of *C. spinarum* to clean the infected and worm infested wounds of livestock. The above mentioned medicinal applications of the root strongly suggest an activity against bacteria. It is therefore of interest to follow up on this lead and screen members of the *Carissa* family for antimicrobial compounds. With respect to *C. lanceolata*, this was one objective of this work.

1.6.1 Assay Methods

Mainly three methods are used in plant drug analysis to screen for and assess antibacterial or antimicrobial activities. The two more conventional methods[65] are disc diffusion and well diffusion assays, where the microbes to be studied are inoculated to semisolid media and test samples are introduced to diffuse into the media. Disc diffusion uses cellulose discs, which are treated with the test sample dissolved in a suitable volatile solvent. The dried discs are kept in contact with the inoculated media to allow diffusion before being incubated to promote bacterial growth. In the well diffusion method, small circular wells are cut into the solidified inoculated media. These wells are then filled with the sample solution using a suitable inert solvent. The solution is allowed to diffuse into the media for a short time before incubation. In both diffusion assay types antimicrobial activity is assessed as a measure of the width of a zone of inhibition of growth around the sample (on the disk or in the well), which can also be correlated to the strength of the test solution to establish minimal inhibitory concentration (MIC) values.

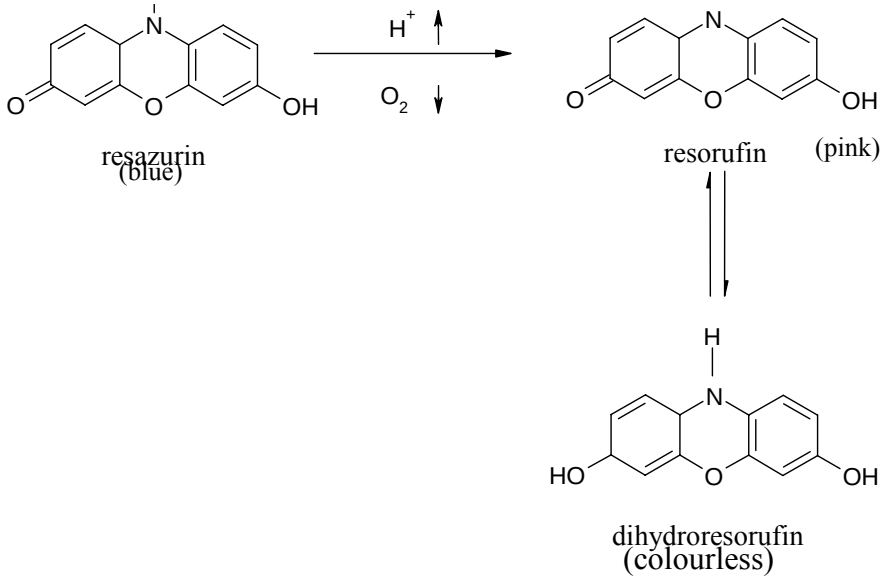
An alternative method used in this study is the micro broth dilution method[66]. Its principle is similar to the above procedures, but it operates on a much smaller scale (volumes in μL and test samples used in μg amounts). The sample is introduced to an inoculated liquid media, in which the compound will be diluted and dispersed. After

incubation of this solution for the respective time, microbial growth can be observed by turbidity or using a suitable dye. In the latter procedure, the intensity of the colour formed can be measured by spectrometry to quantify the antimicrobial activity. In all cases serial dilutions are used to calculate MIC values.

Micro broth dilution was found to be an efficient and reliable method, with the additional advantage of requiring only minimal quantities of test samples. Using 96 well plates (ELISA plates); a serial dilution can also be performed simultaneously for many compounds at one time, allowing a qualitative and a quantitative assay in a single, very time efficient step. As a result, MIC values for many compounds against one particular strain can be obtained from a single experiment and even multiple studies can be performed in the same plate to validate results[66].

Taking into account these advantages, the antibacterial activity of *C. lanceolata* was therefore studied by micro broth dilution assay in this project using the above described microbroth dilution method on 96-well plates. Resazurin was used as indicator for microbial growth, which has frequently being an accurate and convenient method providing qualitative and quantitative results in a single step[66]. In a serial dilution sequence, the margin between growth and inhibition was clear enough to determine the MIC from either bactericidal or bacteriostatic activity, since reduction of blue coloured resazurin to pink colour resorufin was observed as an indication of microbial growth, while a persisting blue colour in the test solution demonstrated antimicrobial activity[66] (Figure 10). This method was found to be superior to the conventional disc diffusion and well diffusion methods[67-70] (Appendix III).

Figure 10: Reduction Path of Resazurin[66]



1.6.2 Bacterial Strains used in the Antibacterial Bioassay

As a member of the Enterobacteriaceae family, morphologically *E. coli* are short rod shaped Gram negative bacteria, which are commonly found as intestinal flora. *E. coli* endotoxins can cause urinary tract infections, diarrhoea, sepsis and meningitis when found outside the intestine. They are sensitive towards a wide range of antibiotics and other antimicrobial agents, but tend to be resistant to β-lactam antibiotics, trimethoprim and vancomycin due to mutations. *E. coli* cultures grown on nutrient agar plates are smooth, circular, domed shape colonies[71].

S. aureus, on the other hand, is a member of the Staphylococci family. Being a Gram positive cocci, it is found on dermal and mucosal flora. *S. aureus* is different to other members of the family insofar as it is the only coagulase positive Staphylococci. It causes aggregation of the bacteria by bonding to fibrinogen so the bacteria cell is less likely to be ingested by phagocytes. β-Lactam antibiotic resistance is evident due to the presence of *mec*A gene; furthermore, it also shows resistance to tetracyclines. Colonies of *S. aureus* appear smooth, round, raised and have a wet look[71].

The genus *Bacillus* includes large aerobic, Gram-positive rods occurring in chains. Most members of this genus are saprophytic organism prevalent in soil, water and air and on vegetation, some are pathogens. Typical cells have square ends and are arranged in long chains, spores are located in the centre of the nonmotile bacilli, its colonies have a cut glass appearance[71].

Pseudomonas, on the other hand, is a Gram negative bacterium, widely distributed in soil and water. *P. aeruginosa* sometimes colonises and causes diseases in humans, but is normally present in intestine flora. It is motile and rod-shaped, occurs as single bacteria, in pairs and occasionally in short chains[71].

2 Materials and Methods

2.1 Plant Material

Plant material was collected in Broome, Western Australia during the month of March 2005 by Mr. Kim Courtenay of Kimberly TAFE. It was sent to the School of Pharmacy, Curtin University of Technology, where a voucher specimen was deposited.

The root bark was separated from the root wood and both samples were dried at 37^{o}C for 24 hrs. The size of the plant particles was reduced to less than 3 mm by Hammer mill (DHF-48, Universal Scientific Company, WA, Australia) using a 3 mm sieve.

2.2 Solvents

The following solvents mentioned in the table 3 were used in the various experiments of this work:

Table 3: List of solvents used in the study

Solvent	Grade	Supplier
Dichloromethane	HPLC	Labscan Asia Co., Ltd.
Hexanes	Analytical Reagent	Mallinckrodt Baker Inc.
Methanol	HPLC	Labscan Asia Co., Ltd.
Ethyl acetate	Analytical Reagent	Selby Scientific.
Acetone	Analytical Reagent	Mallinckrodt Baker Inc.
Petroleum Ether 40 – 70^{o}C	Analytical Reagent	Merck Pty. Ltd.
Ethanol	Analytical Reagent	CSR Distilleries Ltd.
Chloroform	Analytical Reagent	Merck Pty. Ltd.

2.3 Extractions

2.3.1 Steam Distillation

Fresh root bark (13.07g) was subjected to steam distillation. Steam was introduced to an aqueous suspension of the sample, which was maintained at 100^{o}C. Generated vapours were condensed by cooling and collected for 1.5 hrs. As no distinct volatile oil droplets had separated, the aqueous distillate was extracted three times with 25 mL DCM. The organic bottom layer was dried over anhydrous sodium sulphate, the

drying agent filtered off and the solvent carefully evaporated at room temperature. A resultant residual oil of 7.2 mg was collected.

2.3.2 Solid Phase Micro-Extraction (SPME)

One gram of fresh root bark was placed in a vial and a micro fibre was inserted into the vial with the help of a micro-fibre holder (Supelco Lot 248317). This setup was kept at 37^{o}C for 30 min before the micro fibre was introduced into the GC/MS for analysis.

2.3.3 Solvent Extraction

Root wood (58.310 g) and root bark (32.971 g) were separately extracted in a Soxhlet apparatus. Hexane, DCM and MeOH (1L each) were used to extract the samples successively. Extracts obtained were concentrated by rotary vacuum evaporation at 50^{o} C (Rotavapor 200, Büchi, Switzerland), then further dried under vacuum at ambient temperature using a vacuum oven (National Appliances, Oregon, USA). Dried extracts were stored in sealed vials at 4^{o} C until used for further analysis.

2.4 Chemical Tests

Extracts were dissolved in 70% acetone to a final concentration of 50 mg/mL in order to perform the following chemical tests:

2.4.1 Determination of Alkaloids

One millilitre of the test solution was treated with a few drops of Dragendroff's reagent (Appendix II). Another aliquot (1 mL) was treated with a few drops of Mayer's reagent (Appendix II). The formation of a precipitate was seen as indicating the presence of alkaloids[72, 73].

2.4.2 Test for Saponins

One millilitre aliquots of the various plant extracts were combined with 5 mL of 60^{o} C water, then shaken for 2 min. As saponins are known to possess frothing activity[72], the volume of froth produced in this experiment was observed and recorded every 10 min for a total period of 30 min.

2.4.3 Test for Phenolic Compounds

One millilitre of test solution was treated with 10% ethanolic ferric chloride. Phenolic compounds were considered present when a colour change to blue green / dark blue was observed[72, 73].

2.4.4 Test for Anthraquinones

The Bornträger Test[72] was used for the detection of anthraquinones. Two millilitre of the test sample was shaken with 4 mL of hexane. The upper lipophilic layer was separated and treated with 4 mL of dilute ammonia. If the lower layer changed to violet to pink was indicated the presence of anthraquinones.

2.4.5 Test for Steroids

One millilitre of the respective plant extract was treated with three drops of acetic anhydride and one drop of concentrated sulphuric acid. A colour change from deep green turning to brown to dark brown, indicated the presence of sterols[72, 73] .

2.4.6 Cardiac Glycosides

Following the protocol of the Keller-Killiani Test[72, 73], 1 mL of sample solution was mixed with One millilitre of glacial acetic acid then treated with one drop of 5% ethanolic ferric chloride solution. One millilitre of concentrated sulphuric acid was carefully poured down the side of the test tube. The appearance of a brownish ring between the two formed layers with the lower acidic layer turning blue green upon standing indicated the presence of cardiac glycosides.

2.5 Chromatographic Methods

2.5.1 Thin Layer Chromatography (TLC)

Thin layer chromatography was used to profile extracts and collected column fractions and also to analyse the isolates qualitatively by comparing their R_f values with published data. Silica gel F_{254} aluminium backed plates of 20 cm by 10 cm were used for the TLC experiments (Macherey-Nagel GmbH &Co. KG. Duren, Germany). A total of 4 µL of sample solution was either introduced to the plates using a Linomat IV (Camag, Switzerland) autosampler through a 100 µL syringe (Camag, Switzerland), or manually spotted onto the stationary phase using a glass capillary. The following solvent systems were used as mobile phase (table 4):

Table 4: Mobile phases used in TLC

S. No	Solvents	Ratio
1	Hexane : Ethyl acetate	7:3
2	Hexane : Ethyl acetate	5:5
3	Hexane : Ethyl acetate	3:7
4	Pet. Ether (40 -70): Ethyl acetate	3:7
5	Ethyl acetate : Methanol	8:2
6	Chloroform : Methanol	8:2
7	Chloroform : Methanol	9:1
8	Chloroform : Ethyl acetate	1:4
9	Methanol : Ethyl acetate : Water	1:8:1
10	Methanol : Ethyl acetate : Water	2:7:1

Developed plates were dried and assessed at 254 and 366 nm (Alltech UV Cabinet CM10 Spectroline). Anisaldehyde (BDH Chemicals, Poole, England) spray (Appendix II), 5% sulphuric acid in ethanol and iodine vapour were also used as visualisation techniques.

2.5.2 Column Chromatography

DCM and MeOH extracts were further separated by column chromatography using glass columns with sintered glass filters. Silica gel (60-120 mesh, Merck KGaA, Darmstadt, Germany) was loaded as slurry prepared in the respective solvent.

Fractions were collected under gravitational flow. Purification columns were also run in a similar manner using neutral alumina (Sigma Chemicals Co., St. Louis, MO, USA) as stationary phase.

2.5.2.1 Dichloromethane Extracts

An aliquot of the DCM extract of the root bark (1.4 g) was incorporated into 2.8 g of silica gel, and then separated over 141.4 g of silica gel (column length 176 mm, diameter 30 mm, bed volume 210 mL). DCM, EtoAc and MeOH were subsequently used as mobile phases in a gradient system with a 10% increase of each subsequent solvent. Hundred millilitres of each mobile phase was used and 20 mL fractions were collected. The collected fractions were pooled according to their TLC profile (solvent system 4) to obtain five major fractions. Fractions 2 and 3 were further separated as described below.

2.5.2.1.1 Fraction 2

Fraction 2 (134 mg) was dissolved in DCM and wet loaded onto a suitably sized column (length 110 mm, diameter 15 mm, bed volume 20 mL) containing 13.4 g of silica gel as stationary phase. Petroleum ether and DCM were used in a gradient system as mobile phase with a 10% increase of each subsequent solvent. Twenty millilitre of each mobile phase were used and 10 mL fractions were collected. Five distinct fractions were identified and the second of those fractions was further separated by preparative TLC.

2.5.2.1.2 Fraction 3

A total of 209 mg of Fraction 3 were dissolved in DCM (2 mL) and wet loaded onto 20.9 g of silica gel (column length 205 mm, diameter 15 mm, bed volume 31 mL). DCM and ethyl acetate were used as mobile phase in a gradient system with a 10% increase where 20 mL of each mobile phase was used, followed by an isocratic system of 100 mL of EtoAc and ethanol (1:1). Ten millilitres fractions were collected from both systems and pooled according to their TLC profiles to obtain three major fractions. The second of those fractions was separated on a preparative TLC plate.

2.5.2.2 Methanol Extracts

A MeOH extract of the root wood (3.674 g) was separated over 100 g of silica gel after incorporating the sample into 7.35 g of silica gel (column length 150 mm, diameter 30 mm, bed volume 150 mL). EtoAc and MeOH were used as mobile phase in a gradient system with a 10% increase. Hundred millilitres of each mobile phase was used and 20 mL fractions were collected. Four distinct fractions (Fractions 1-4) were identified.

The first fraction (0.846 g) was dissolved in 3 mL of MeOH then wet loaded and separated by chromatography over 30 g of silica gel (column length 280mm, diameter 15 mm, bed volume 45 mL) using MeOH:DCM (1:1) as mobile phase. The fraction of interest (0.641 g) was eluted with EtoAc: DCM (4:1) over 30 g of silica gel, introduced as a solution in 2 mL of MeOH (column length 280mm, diameter 15mm, bed volume 45 mL). The fraction of interest (0.444g) was further purified on 10 g of silica gel (column length 100mm, diameter 15mm, bed volume 15 mL) with MeOH: DCM (98:2) followed by another column chromatography on 2g of silica gel (column length 50 mm, diameter 7 mm, bed volume 3 mL) with EtoAc: DCM (1:1) as mobile phase.

2.5.2.3 Hexane Extracts

2.5.2.3.1 Root Bark Hexane Extract

A hexane extract (934 mg) was incorporated into 1.87 g of silica gel then introduced to 45 g of silica gel using a suitably sized column (length 70 mm, diameter 30 mm, bed volume 67 mL). It was separated using a gradient system of hexane, DCM and EtoAc as mobile phase with a 10% increase of each subsequent solvent. Sixty millilitres of each mobile phase were used and 20 ml fractions were collected. After pooling various fractions based on an identical TLC profile (Solvent system 4), Fraction 2 was further separated on 25 g of silica gel by wet loading (column length 220 mm, diameter 15 mm, bed volume 31 mL) using DCM with increasing concentrations of EtoAc (5% gradient) as mobile phase. The isolated compound was further purified over a 5 g of alumina column (column length 75 mm, diameter 7 mm, bed volume 15 mL) using DCM as mobile phase.

2.5.2.3.2 Root Wood Hexane Extract

Total of 1.14 g of root wood hexane extract was incorporated in 2.28 g of silica gel, then loaded onto 114 g of silica gel (column length 143 mm, diameter 15 mm, bed volume 171 mL), 20 mL each were collected from DCM with increasing amounts of EtoAc (5% per gradient step) as mobile phase. After studying their TLC profiles (Solvent system 4), the fractions were pooled to obtain three major fractions. The third fraction was separated subsequently by column chromatography on 20 g of silica gel (column length 200 mm, diameter 15 mm, bed volume 30 mL) using DCM as mobile phase. The compound isolated from this procedure was further purified with EtoAc over an alumina column (alumina 5g, column length 75 mm, diameter 7 mm, bed volume 15 mL).

2.5.3 Preparative TLC

Preparative TLC plates (Analtech Uni Plate, Silicagel G, 400 cm^2) of 2000 μm thickness were used in this study. Samples (100 mg/mL) were spotted onto the preparative TLC plate using a Linomat IV auto sampler through a CAMAG 500 μL syringe. Plates were developed in Solvent System 4 as mobile phase. A 1cm wide area of the developed chromatogram was exposed to anisaldehyde spray and heated in a hot air stream. The rest of the plate was covered with aluminium foil and a ceramic tile was placed on top for insulation to visualise the separation. The corresponding untreated areas of the remainder of the plate were scraped off and the compounds extracted from the stationary phase with a suitable solvent.

This technique was employed to separate compounds from *Fraction 2* of the root bark DCM extracts, which had in an earlier step been obtained by column chromatography (Section 2.5.2.1.1). Fraction of 50 mg containing the compound of interest was dissolved in 500 μL of DCM and introduced to the preparative TLC plate. After development, the compounds on the plate were visualised, the band containing the compound of interest was scraped off and dispersed in DCM. The dispersion was filtered and the solvent evaporated to obtain the pure compound.

In total, 88 mg were obtained from Fraction 3 obtained after column chromatography of the root barks DCM extract. Total 50 mg of the sample was dissolved in 500 μL of DCM and then introduced to a preparative TLC plate. A compound of interest was isolated in the same manner as described above using DCM as the solvent to extract from silica gel.

2.5.4 Gas Chromatography

Gas chromatography-mass spectroscopy was used to identify those isolated compounds, which were thermostable and volatile using a Hewlett Packard 5890 instrument with a HPMS 5 Column (Agilent Technologies, 30 m length, 0.25 mm internal diameter).

Helium was used as the mobile phase with a flow rate of 0.9 mL/min under a pressure of 7.0 psi. The injection port temperature was kept at 120^{o}C with the starting temperature of the column set at 60^{o}C increasing to a final temperature of 250^{o}C over a period of 26 min. The detector temperature was kept at 280^{o}C.

2.6 Spectroscopy

2.6.1 Infrared Spectroscopy (IR)

Fourier transformation infrared spectroscopy (Perkin-Elmer Spectrum One) was used for spectroscopic analysis of some samples. These were dissolved in DCM or acetone then loaded onto sodium chloride discs (500A3 NaCl, Buck Scientific, CT, USA) and dried before their IR spectrum was recorded.

2.6.2 Mass Spectroscopy (MS)

Fractions separated by GC were detected by a Hewlett Packard Mass Selective Detector 5971. The detected compounds were identified by HP Chem Search Libraries (Wiley 275) as well as by careful analysis of the obtained fractionation pattern.

2.6.3 Nuclear Magnetic Resonance Spectroscopy (NMR)

Preliminary ^{1}H NMR spectra were recorded on a Varian Gemini 200 MHz NMR spectrometer. More detailed ^{1}H and ^{13}C NMR spectra of selected compounds were provided by Dr. Lindsay Byrne (School of Biomedical, Biomolecular and Chemical sciences, University of Western Australia) using a Bruker Advance 500MHz NMR spectrometer. Samples were dissolved in deuterated solvents such as d-chloroform (Cambridge Isotopes, Andover, MA, USA) and d-acetone (Sigma-Aldrich Chemicals St. Louis, MO, USA)

2.7 Antimicrobial Assay

Antimicrobial assays were carried out in the Microbiology Facilities within the School of Pharmacy, Curtin University of Technology. Aseptic conditions were maintained using sterile equipment and substances.

2.7.1 Microbial strains

Bacterial strains used in the study were selected based on their availability and previous use reported in similar studies. The selected strains were *Escherichia coli* (W), *Bacillus subtilis* (ATCC 6633), *Pseudomonas auriginosa* (ATCC 19429) and *Staphalococous aureus* (ATCC 6538P).

2.7.2 Assay Material

Microbial assays were performed on 96-well plates (Polystyrene High Transparency) supplied by Bioster s.p.a, PD, Italy. Nutrient broth was used as the media (Oxoid Ltd. Basingstoke, Hampshire, England) and resazurin sodium (obtained from BDH Chemicals, Poole, England) as indicator. Water used in the experiments was filter purified with MilliQ Filters then autoclaved for sterilisation. Streptomycin sulphate (Glaxo Australia Pty. Ltd. VIC Australia) was employed as the positive control. Extracts and isolates were dissolved in 70% acetone with 0.2% polyoxyethylenesorbitan monooleate (Tween 80) (Sigma Chemicals St. Louis, MO, USA) as a suspending agent.

2.7.3 Assay Method

Liquid media was inoculated with a single colony then incubated over night before being diluting 10 fold with sterile saline solution. Stock solutions of each extract and fraction (100 mg/mL) were prepared using 70% acetone with 0.2% Tween 80 as solvent mixture. Serial dilutions were then carried out in the wells as outlined (Table 5) to obtain the given concentrations.

Table 5: Volume of media and sample in wells

Volume of 70% Acetone (µL)	Volume of Extract (µL)	Volume of Culture (µL)	Concentration (mg/ mL)
0	20 (blank solvent)	60	25
0	20	60	25
4	16	60	20
8	12	60	15
12	8	60	10
16	4	60	5
18	2	60	2.5
19	1	60	1.25
20	0	60	0

All assays were performed in triplicate and separate plates were used for each strain to avoid cross-contamination. Streptomycin 10 mg/mL was used as a positive control and pure solvent as negative control.

Plates were incubated for 18 hrs at 37^{o}C. Then, 120 µL of sterile saline solution was added to each well to dilute the concentration to enable visualisation of any subsequent colour change. Volume of 5µL 0.01 % resazurin sodium solution (in Milli-Q water) was added to each well and the plates incubated for 60 min. The reduction of the dye with subsequent colour change from blue to pink was observed in samples with bacterial activity. Blue coloured wells indicated an antibacterial activity, and allowed also the determination of the MIC of the respective compound.

3 Results & Discussion

3.1 Extraction

Table 6 lists the amounts of extracts obtained in the various extraction procedures with the percentage yield calculated with reference to the dried plant material used.

Table 6: Extracts and their percentage yield

Plant source	Solvent	Amount (in g)	Percentage Yield	Extract Number
Root Bark (32.971 g)	hexane	0.528	1.60%	3
	dichloromethane	0.456	1.38%	4
	methanol	5.250	15.92%	5
Root Bark (175.080 g)	hexane	0.934	0.53%	3
	dichloromethane	1.414	0.81%	4
Root Wood (58.310 g)	hexane	1.115	1.91%	6
	dichloromethane	0.558	0.96%	7
	methanol	4.224	7.24%	8

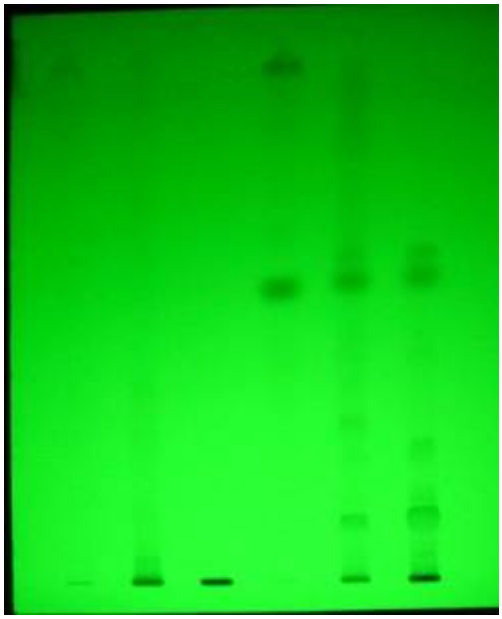
As is apparent from Table 6, MeOH extracts were highest in yield. A second larger scale extraction of root bark using hexane and DCM was carried out to obtain more material for the various separation and isolation procedures carried out in this project.

3.2 Preliminary phytochemical studies

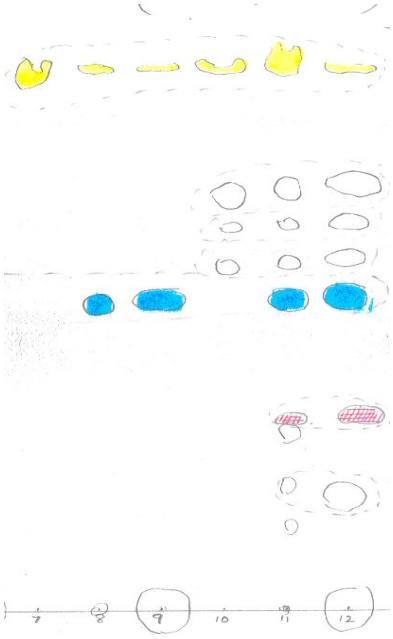
3.2.1 TLC screening

Extracts were studied for their TLC pattern with different solvent systems. The most efficient mobile phase system for hexane and DCM extracts was found to be EtoAc: petroleum ether (7:3). The chromatograms observed under UV_{254} and UV_{366} (TLC-1) and after visualisation with anisaldehyde spray (TLC-2) (Figure 11). MeOH extracts demonstrated the best resolution of compounds using an EtoAc : chloroform (4:1) solvent system for their moderately polar compound fraction (TLC-3), while more polar substances were separated best by an EtoAc : MeOH : water (7:2:1) mixture (TLC-4).

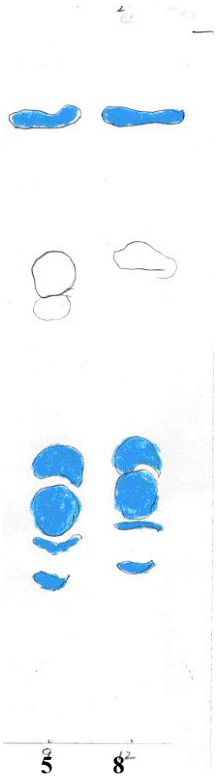
Figure 11: TLC in different solvent systems and visualisations



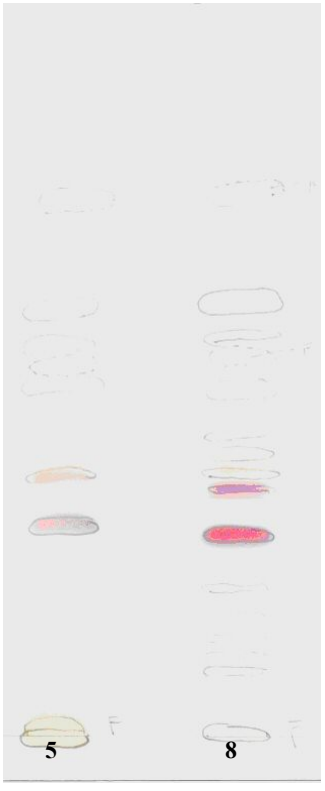
(TLC-1)



(TLC-2)



(TLC-3)



(TLC-4)

3.2.2 Chemical tests

Basic chemical tests were performed to identify the presence / absence of certain compound classes in the various extracts. As can be seen from the obtained results (Table 7), root wood and bark appear to contain alkaloids, which are extractable by MeOH what might indicate a polar character. Phenolic compounds were demonstrated in MeOH and, to a lesser extent, also in the DCM extract of root wood as well as in the MeOH extract of root bark material. MeOH extractable saponins were found to be present in the root bark. Test for steroids were positive for both wood and bark MeOH extracts, while only wood extracts were positive for cardiac glycosides. None of the extracts tested positive for anthraquinones.

Table 7: Chemical tests

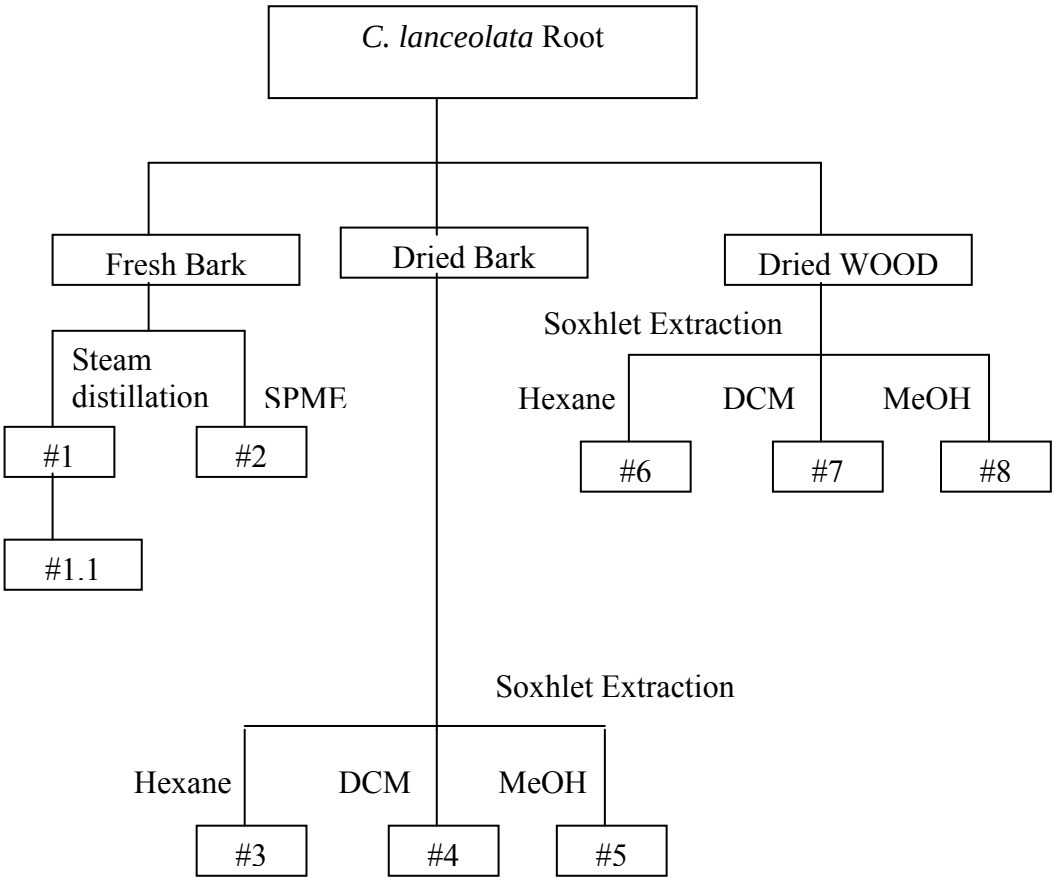
Morphological part	Solvent	Alkaloids		Phenolic Compounds	Cardiac - glycosides	Steroids	Saponins	Anthraquinones
		Dragendorff's	Mayor's	$FeCl_3$	Keller-Kiliani	Liebermann – Burchard Test	Froth Test	Bornträger's Reaction
Root bark	Hexane	Nil	Nil	Nil	Nil	Nil	Nil	Nil
	DCM	Nil	Nil	Nil	Nil	Nil	Nil	Nil
	Methanol	+++	++	++++	Positive	Positive	2mL in 0 min 1mL in 10 min	Nil
Root wood	Hexane	+	Nil	Nil	Nil	Nil	Nil	Nil
	DCM	+	+	+	Positive	Positive	Nil	Nil
	Methanol	++++	+++	+++	Positive	Positive	Nil	Nil

+ - faint, ++ - clear, +++ - very clear, ++++ - highly intense

3.3 Volatile oil [#1]

Steam distillation of 13.07 g of fresh root bark provided 7.2 mg of volatile oil (Figure 12). The total percentage of oil was thus found to be 0.55%. The sample obtained from this procedure as well as the parallel conducted experiment of solid phase micro extraction (SPME) of fresh root material by micro fibre technique [#2], were analysed by GC/MS. Gas chromatography trace and mass spectra for both extracts suggested a single compound. Careful analysis of its fragmentation pattern in combination with comparison of the obtained data with the HP Chem©, Wiley 275 ©library identified the compound as 2′-hydroxy acetophenone (#1.1) (CAS number 118-93-4) with a 91% level of confidence. ^{1}H NMR analysis further confirmed this structure (see Section 3.7.1).

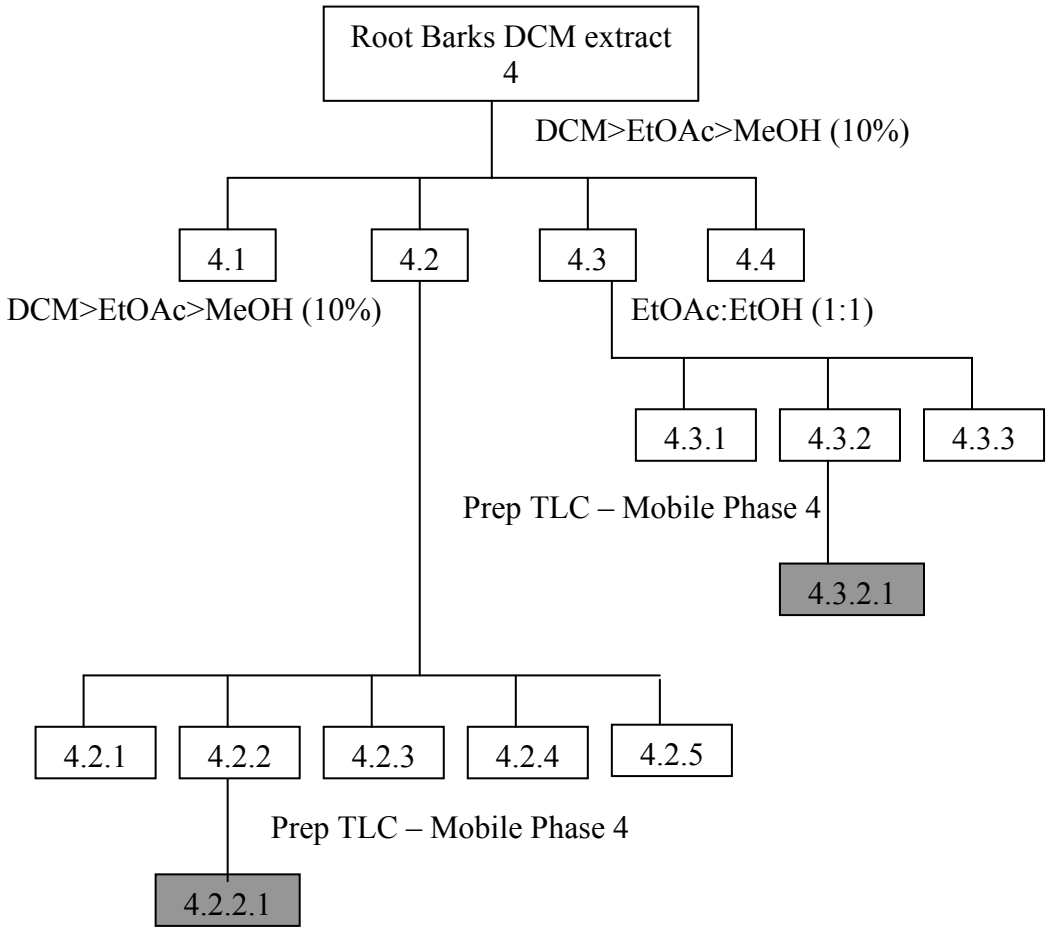
Figure 12: Isolation path of the C. lanceolata root



3.4 Dichloromethane Extracts [#4]

DCM extracts of the root bark were selected for this study, as the root wood's DCM extracts exhibited a TLC pattern similar to the corresponding MeOH extracts. A sample of extract (1.414 g) was separated on silica gel by column chromatography using a solvent gradient with increasing polarity (DCM, EtoAc to MeOH in 10% steps). After pooling based on the TLC pattern obtained with Mobile System 4, four distinct fractions were obtained of which Fraction 2 and Fraction 3 were further studied (Figure 13).

Figure 13: Isolation path for DCM extracts



3.4.1 Fraction 2 [#4.2]

A total of 134 mg of Fraction 2 was again subjected to column chromatography on silica gel using the same mobile phase system (DCM, EtoAc to MeOH in 10% steps) to obtain five fractions. The second fraction [#4.2.2] was initially tried to be further purified by column chromatography using increasing concentrations of DCM in petroleum ether as mobile phase and silica gel stationary phase. Since this procedure was unsuccessful in separating the constituting compounds, preparative TLC was performed as the compounds were sufficiently resolved when developed in Mobile Phase 4. A volume of 500 µL of a 50 mg/mL sample was spotted on to the preparative TLC plate, which was then developed in a vapour-saturated chamber using EtoAc : petroleum ether (7:3) as mobile phase. A major spot quenching at 254 nm with a purplish appearance upon spraying with anisaldehyde reagent was identified as the sole compound of this fraction [#4.2.2.1]. Other solvent systems were also attempted (e.g. Mobile Phases 1, 7, 8 and 10), but a single spot was always obtained, hence it was concluded that this fraction consisted of only one relatively pure compound. Similarly, a single peak at a retention time of 16.64 min was also obtained on GC/MS using the GC method discussed in Section 2.5.4. However, MS data did not help with structural elucidation. The isolated compound (11.3 mg) was therefore also subjected to IR, ^{1}H and ^{13}C NMR spectroscopy for structural identification. Based on these analyses, the compound was identified as carissone, corresponding to information provided in the literature[43, 58] for this compound.

3.4.2 Fraction 3 [#4.3]

Non-polar solvents systems using varying concentrations of DCM and EtoAc as mobile phase were not able to separate any of the compounds present in the 209 mg sample. However, a mixture of EtoAc and ethanol (1:1) eluted the compounds from silica gel as three major fractions. The second fraction (88 mg) [#4.3.2], which contained two major compounds was further separated by preparative TLC with ethyl acetate: petroleum ether (7:3) using two separate plates and applying 500µL of a 100mg/mL concentrated solution to each. The spot with the lower R_f value (0.45) produced a high intensity blue florescence when observed at 366 nm. After its isolation, this compound (8.2 mg) remained a single spot when analysed in different TLC solvent systems (Table 4). Gas chromatography trace did not show any peaks

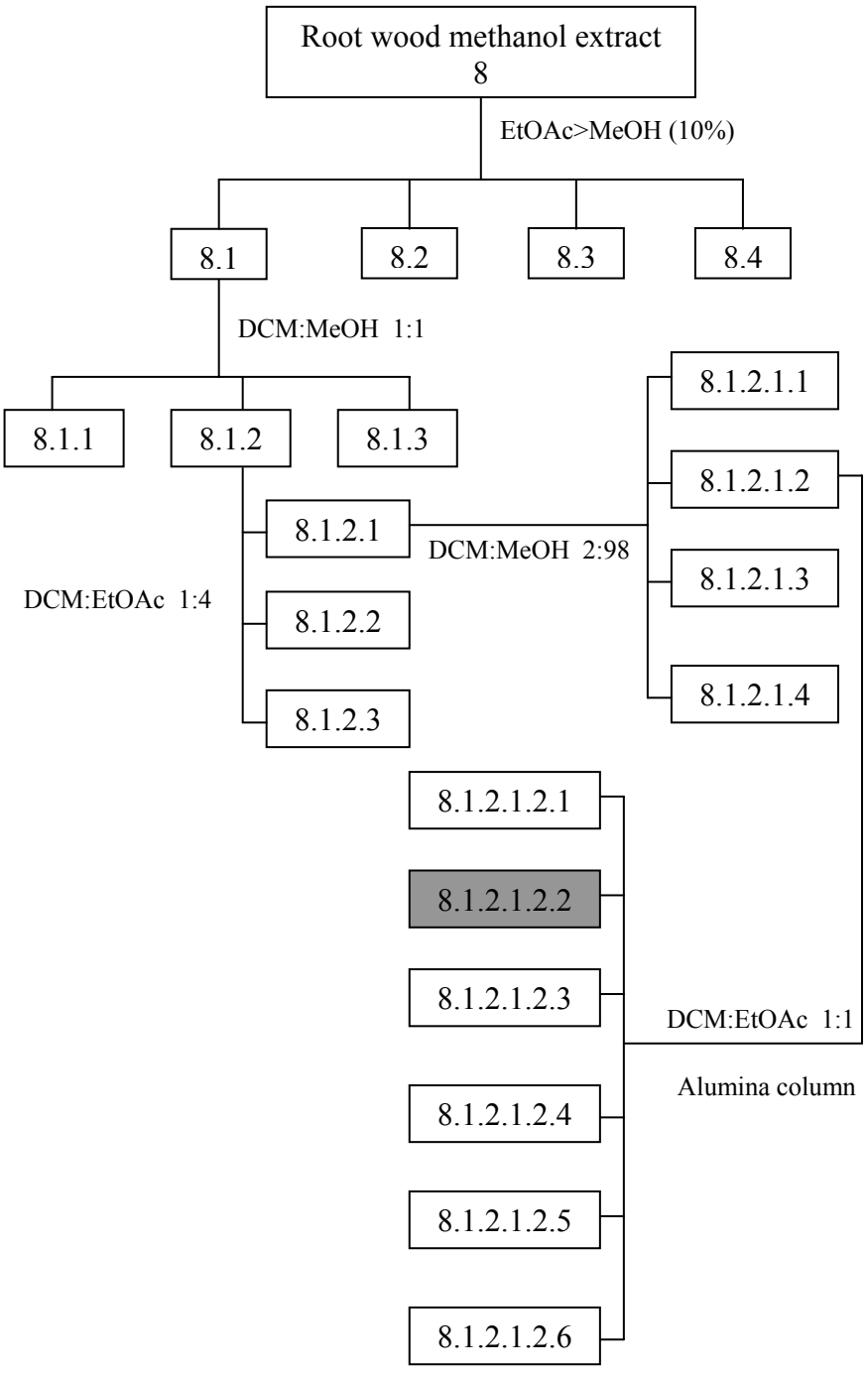
from the sample, hence it appears to be non-volatile in nature. The sample was analysed by FTIR, ^{1}H and ^{13}C NMR spectroscopy for structural identification. The compound showed many spectroscopic characteristics of a eudesmane type sesquiterpene. For instance, ^{13}C signals for the ketone carbon C-3, as well as the methyl groups C-12 and C-15, compare well with other reported eudesmanes reported in section 1.5.1, while the obtained infrared spectrum also showed similarities with other eudesmanes isolated from *Carissa* ssp. The exact structure of the compound [#4.3.2.1], however, is yet to be identified. The amount recovered was insufficient to follow up with further, more detailed structural analysis. Further studies and structural elucidation will therefore be an interesting extension of the current work.

3.5 Methanol extract [8]

Tis study mainly concentrated on MeOH extracts of the root wood (Figure 14), since they appeared to be abundant in lignans. This was indicated by spraying several TLC plates of these extracts with 5% sulphuric acid, which upon heating turned many of the identified spots bright red[28].

Using column chromatography, 3.674 g of total MeOH extract of root wood was separated over silica gel using EtoAc with increasing MeOH concentrations (10% gradient) as mobile phase. After pooling based on identical TLC profiles, four fractions were obtained, of which Fraction 1 [#8.1] was further separated using an isocratic system of DCM : MeOH (1:1) as mobile phase. Working on the second [#8.1.2] of the three major fractions obtained in this procedure, another column chromatography using DCM: EtoAc (1:4) as mobile phase was carried out. A fraction [#8.1.2.1] containing two major lignans was obtained from these experiments. Using MeOH: DCM (2:98) as mobile phase a single compound was obtained by yet another column chromatography experiment from its second fraction [#8.1.2.1.2], which was further purified by DCM: EtoAc (1:1) over alumina. The isolated compound (6.2 mg) was found be a single substance [#8.1.2.1.2.2], as confirmed by various TLC experiments conducted in different mobile phases (see Table 4). It was subjected to ^{1}H NMR and IR analysis. The obtained spectra matched data provided in the literature[28, 44] for carinol. The R_f value of 0.38 (0.36-0.39) in chloroform: EtoAc (1:4) was reported also in the literature[28].

Figure 14: Isolation path of methanol extracts



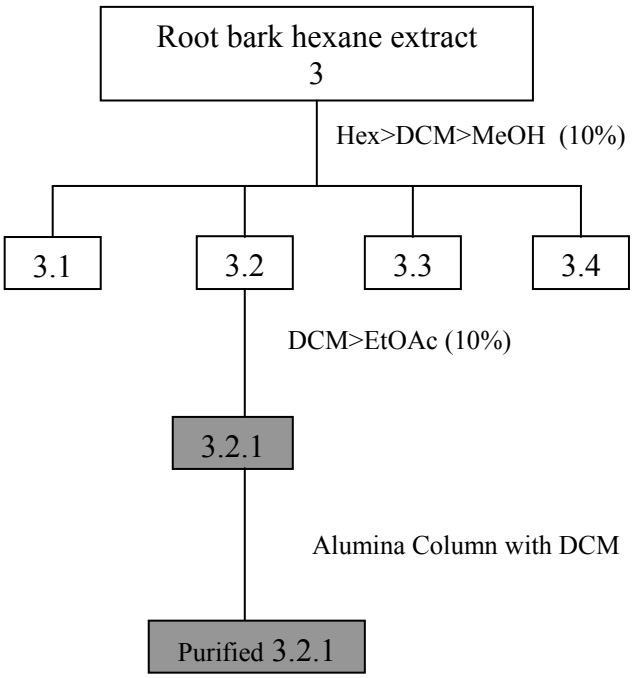
3.6 Hexane extracts

Hexane extracts of root wood and bark were studied separately.

3.6.1 Root bark hexane extract [#3]

The extract (934 mg) was separated by column chromatography (Figure 15) using a gradient system with increasing polarity (hexane, DCM, EtoAc increasing 10% for each fraction). After pooling based on their TLC profiles, four fractions were obtained, of which Fraction 2 (256 mg) [#3.2] was further analysed. When eluted over silica gel, using DCM with increasing concentrations of EtoAc as mobile phase, a single compound [#3.2.1] was isolated (61.1 mg), which was purified further by DCM on alumina. When studied by TLC in changing mobile phases (Table 4), a single spot with a deep green florescence was observed. Spectroscopic analysis and GC/MS data indicated a compound of a complex structure, which could not be identified within the timeframe of this project. The sample was retained for future studies.

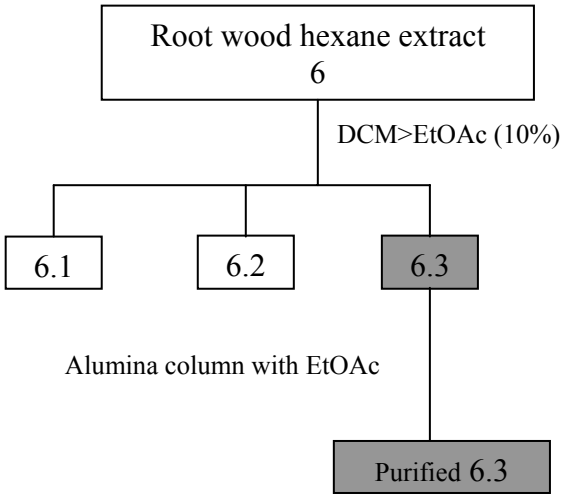
Figure 15: Isolation path of root bark hexane extract



3.6.2 Root wood hexane extract [#6]

An extract of 1.14 g was separated over silica gel by column chromatography using a gradient of DCM with increasing concentrations of EtoAc as mobile phase. After pooling based on identical TLC profiles, three major fractions were identified, the third [#6.3] being similar to compound [#4.2.2.1]. The sample was purified by column chromatography over alumina using EtoAc as mobile phase. The resulting single compound was further purified over alumina using DCM as the mobile phase. Analysis in different TLC systems (Table 4) and GC/MS analysis confirmed this compound (3.1 mg) [#6.3] to be pure. Date obtained byGC/MS and ^{1}H-NMR, identified the compound as carissone with data matching information provided in the literature[43] as well as those obtained from compound [# 4.2.2.1] isolated from the root wood DCM extract (Section 3.4.1).

Figure 16: Isolation path of root wood hexane extract



3.7 Isolated compounds

3.7.1 2′-Hydroxy acetophenone [#1.1]

The volatile liquid obtained by steam distillation and solid phase extraction was identified with a high level of confidence (91%) by the HP Chem Search Libraries (Wiley 275) as 2′-hydroxy acetophenone (Figure 17-18). This compound has previously been isolated from *C. edulis* by Bentley *et al.* (1954)[45] and is also commercially available (Aldrich). Obtained data from ^{1}H NMR of the isolate were compared with this reference material and found to be identical. According to its MS data, the molecular weight of the substance was found to be 136, which also corresponds to the proposed molecular formula of $C_8H_8O_2$. When treating an ethanolic solution of the compound with 10% ethanolic ferric chloride, a deep blue coloration confirmed the presence of a phenolic group. With 2, 4-dinitrophenylhydrazine hydrochloride in MeOH, a yellow colour was formed, indicating the presence of a keto functional group.

Figure 17: GC trace of volatile oil

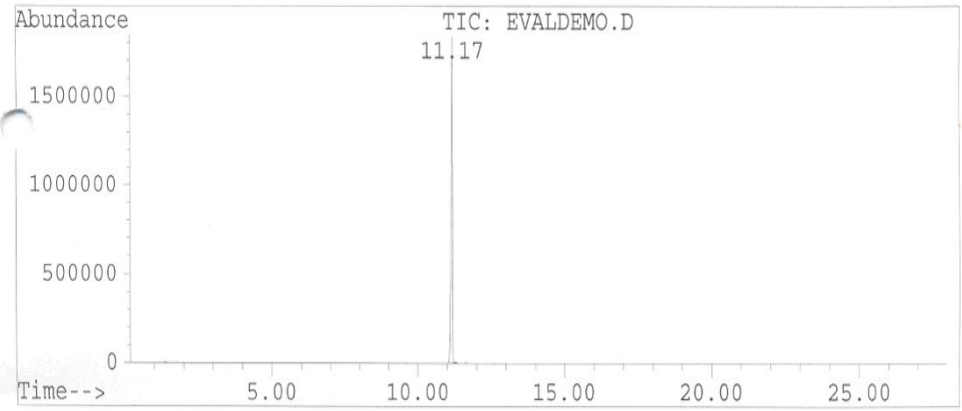
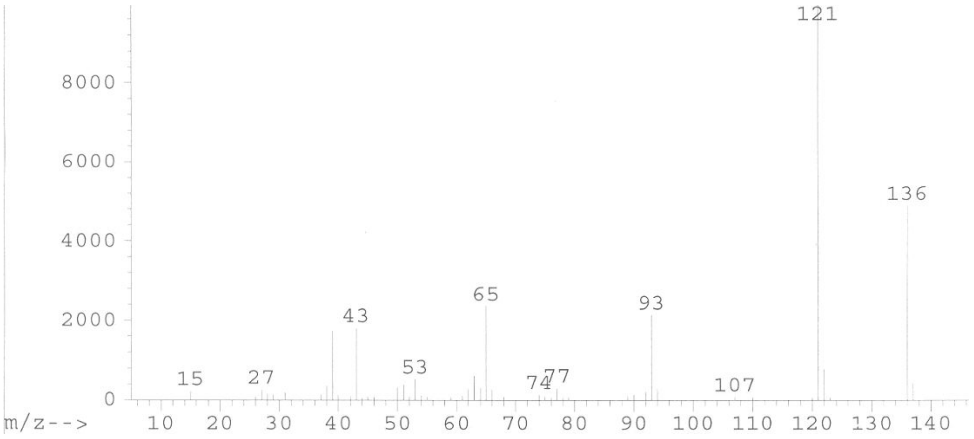


Figure 18: Mass spectra for the volatile compound



The fragmentation pattern of the molecule corresponds with the proposed structure (Figure 19):

Fragment *m/z* 136 represents the intact molecule.

Loss of a methyl fragment resulted in the base peak of *m/z* 121 .

The observed *m/z* 93 corresponds to the phenolic fragment whereas the plain aromatic ring is evident in fragment *m/z* 77.

Figure 19: Fragmentation pattern of 2'-hydroxy acetophenone

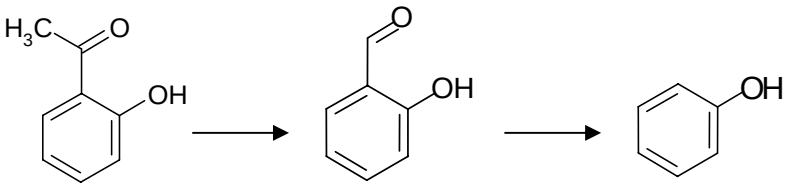


Figure 20: Structure of 2′-hydroxy acetophenone

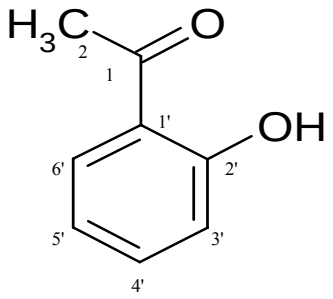


Table 8: ^{1}H NMR spectral signal assignments for 2′-hydroxy acetophenone (Figure 20)

Location of Proton	Number of Protons	δH (ppm)	Appearance of signal
2	3	3.6	s
3′	1	7.7	d
4′	1	7.4	t
5′	1	6.8	dd
6′	1	6.9	d
OH	1	12.2	s

There were 4'-, 2'- and 3'-hydroxy substituted acetophenones have been reported from various plant sources[81]. Confirmation of an ortho-substitution pattern for the acetophenone derivative isolated in this work can be found in the compound's splitting pattern in its ^{1}H NMR spectrum (Table 8). If the compound were to be meta substituted, a singlet for H-2', duplets for H-4′ and H-6′, as well as a duplet of a duplet for H-5′, would have been observed. A para-hydroxyl group, on the other hand, would have resulted in a symmetrical molecule with identical signals for H-2′ and H-6' (duplets) as well as for H-3′ and H-5′ (duplets). The obtained spectrum, however, shows duplets for H-3′ and H-6′ and duplet of duplets for H-4′ and H-5′, which confirms the structure of an asymmetric ortho-substituted acetophenone.

3.7.2 Carissone [#4.2.2.1 and #6.3]

Carissone was previously isolated from *C. lanceolata* root material by Mohr *et al.* in 1954 and also from wood samples by Lindsay *et al.* (2000)[3]. Achenbach *et al.* (1985)[43] and Rastogi *et al.* (1967)[29] in their work on *C. edulis* and *C. carandas* respectively have also reported the presence of this compound. The purified carissone obtained from this work had a colourless, amorphous appearance. Its R_f value in petroleum ether : EtoAc (3:7) was found to be 0.62-0.66, matching data (0.65-0.68) provided by Lindsay *et al*. (2000)[3]. Spectral data obtained by ^{1}H and ^{13}C NMR as well as FTIR spectroscopy for this compound were all in line with data reported by Achenbach *et al.* (1985)[43] and Sathe *et al.* (1971)[58] for carissone.

Figure 21: Structure of Carissone

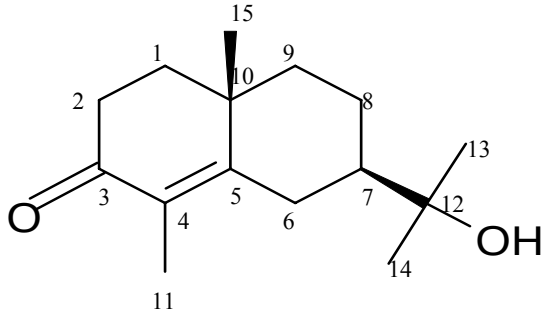


Table 9: ^{1}H and ^{13}C NMR spectral assignments of Carissone (Figure 21)

Carbon	δC (ppm)	Number of Protons	δH (ppm)	Appearance of signal
1	37.31	2H	nic	
2_{ax}	33.76	1H	2.51	ddd
2_{eq}		1H	2.38	ddd
3	199.14	-	nic	
4	128.82	-	nic	
5	162.75	-	nic	
6_{ax}	28.72	1H	1.90	dd
6_{eq}		1H	2.85	ddd
7	49.59	1H	nic	
8	22.55	2H	nic	
9	41.91	2H	nic	
10	35.83	-	nic	
11	10.87	3H	1.77	d
12	72.43	-	nic	
13	26.69	3H	1.25	s
14	27.47	3H	1.24	s
15	22.42	3H	1.19	s

(nic : Not indicated clearly)

3.7.3 Carinol [8.1.2.1.2.2]

Carinol was first reported by Pal *et al.* (1975)[28] from *C. carandas*. Studies on *C. edulis* by Achenbach *et al.* (1983)[44] have also revealed the presence of this lignan. Carinol isolated in this work was amorphous in nature and slightly brown in colour. TLC studies confirmed a R_f value of 0.38 in EtoAc: chloroform (4:1) in line with information published[28]. Data obtained by ^{1}H NMR spectroscopy and FTIR analysis correspond to the spectral values reported[28, 44].

Figure 22: Structure of Carinol

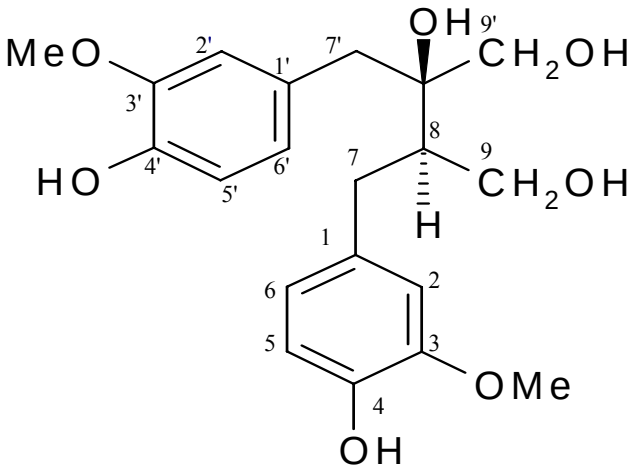


Table 10: ^{1}H and ^{13}C NMR spectral assignments of Carinol (Figure 22)

Proton	Number of protons	δH (ppm)	Appearance of signal
2	1	6.913	d
2′	1	6.909	d
5	1	6.871	-
5′	1		
6	1	6.825	dd
6′	1	6.733	dd
7_A	1	2.588	-
7_B	1		
$7'_A$	1	3.000	-
$7'_B$	1		
8	1	2.053	
9_A	1	3.709	ddd
9_B	1	3.737	ddd
$9'_A$	1	3.53	dd
$9'_B$	1	3.553	dd
O-Me	3	3.83	-
O-Me′	3		
Ar-OH	1	7.35	s
Ar-OH′	1	7.33	s
OH-8′	1	3.61	s
OH-9	1	4.42	dd
OH-9′	OH	4.22	dd

3.8 Antibacterial activity

3.8.1 Antibacterial activity of extracts

Antibacterial assays were performed in triplicates and the average of the obtained minimum inhibitory concentration is listed in Table 11. Very low MIC values signal high antibacterial activity. Where the extracts' MIC was higher than 25 mg/mL, these were considered inactive[65].

Table 11: Minimum inhibitory concentrations of crude extracts

Extract	*P. aeruginosa*	*E. coli*	*S. aureus*	*B. subtilis*
Root bark hexane	20 mg/ml	no inhibition	no inhibition	20 mg/ml
Root bark DCM	25 mg/ml	no inhibition	no inhibition	5 mg/ml
Root bark methanol	2.5 mg/ml	15 mg/ml	25 mg/ml	2.5 mg/ml
Root wood hexane	20 mg/ml	20 mg/ml	15 mg/ml	25 mg/ml
Root wood DCM	2.5 mg/ml	10 mg/ml	10 mg/ml	2.5 mg/ml
Root wood methanol	20 mg/ml	15 mg/ml	15 mg/ml	5 mg/ml

Lipophilic constituents of the root bark appear to be less active against the bacterial strains studied. The root bark DCM extract exhibited, however, considerable activity against *B. substilis* and also some activity against *P. aeruginosa*. No inhibition was observed with *E. coli* and *S. aureus*. MeOH extracts of bark were also active, indicating the presence of polar antimicrobial compounds. Compared to root bark extracts, root wood extracts displayed stronger antibiosis with the DCM extracts of the root wood possessing the highest activity. Considering that the above extracts are all multi-compound mixtures, the observed antibacterial activity demonstrated by these crude extracts is considerable. In general, all the extracts displayed antibacterial activity regardless of the bacterial strain used for the analysis. Overall, Gram negative bacterium, *B. subtilise*, however, was found to be more susceptible to the

extracts, whereas the Gram positive *S. aureus* and Gram positive *E. coli*, showed the maximum resistance to the studied extracts.

3.8.2 Antibacterial activity of isolated compounds

Isolated compounds were also tested for their antibacterial activity (Table 12) by using serial dilutions of 10mg/mL to 1.25 mg/mL. A minimum inhibitory concentration above 10 mg/mL was not considered antibacterial[65].

Table 12: Minimum inhibitory concentrations of the isolated compounds

Compound	*Ps. aeruginosa*	*E. coli*	*S. aureus*	*B. subtilis*
2'-hydroxy acetophenone	1.25 mg/ml	1.25 mg/ml	1.25 mg/ml	1.25 mg/ml
carinol	No inhibition	1.25 mg/ml	1.25 mg/ml	1.25 mg/ml
carissone	No inhibition	5 mg/ml	10 mg/ml	5 mg/ml

The chemical diversity of the isolated compounds makes it difficult to directly compare their activity against the different bacterial strains used. However, it appears that the phenolic volatile compound, 2'-hydroxy acetophenone, is the most active antibacterial compound of the three isolates. The lignan carinol was also active against all the studied strains with the exception of *P. aeruginosa*. The sesquiterpene carissone was the least active in direct comparison, yet produced significant activity. The study also illustrated that Gram positive *P. aeruginosa* was more resistant towards the isolated compounds compared to the other three strains tested. The other Gram positive bacterium, *S. aureus,* demonstrated more resistance than the two Gram negative strains, *E. coli* and *B. subtilis*, which showed the highest sensitivity against the studied compounds.

The antibacterial activity of the isolated compare did not correspond well with that observed for the crude extracts. All the crude extracts inhibited *P. aeruginosa,* but of the three isolated and identified compounds, only 2'-hydroxy acetophenone was found to posses an activity against the growth of *P. aeruginosa*. *E. coli* and *S. aureus*

were resistant against the root bark's DCM and hexane extracts, while carissone, which was isolated from the DCM and hexane extracts of the root bark, exhibited significant activity against these two strains. These complex findings simply illustrate that antibacterial activity obtained from crude extracts is very difficult to evaluate as they constitute multi-compound mixtures. The observed antibacterial activity of an extract ultimately depends on the concentrations of its antibacterial constituent(s). The antibacterial testing of isolated compounds therefore gives a much more accurate reflection of any antibacterial activity present.

Results from these antibacterial assays confirm that *C. lanceolata* root bark and wood both contain various antibacterial compounds found in the volatile oil, less polar lipophilic as well as moderately polar hydrophilic extracts and might therefore substantiate some of the traditional medicinal claims of this plant.

4 Conclusion

Carissa lanceolata root exhibited considerable anti bacterial activity against both Gram negative and Gram positive organisms. Crude extracts of its bark and wood, which were analysed separately, demonstrated that polar compounds were more active against the four strains of bacteria studied in this project. The root bark was found to contain a volatile oil, isolated by steam distillation as well as solid phase micro extraction. It consisted of a single compound, which was identified as 2'-hydroxy acetophenone. The identity of this compound was confirmed by GC/MS and ^{1}H NMR spectroscopy. After its isolation and identification, 2'-hydroxy acetophenone was also tested for its antibacterial properties and, not surprising considering its phenolic nature, was found to be highly active (MIC 1.25mg/mL – against all the four studied strains).

From the root bark's DCM extract, the eudesmane, carissone, was isolated by a series of chromatographic experiments and its identity was confirmed by IR, ^{1}H and ^{13}C NMR spectroscopy. In the antibacterial assays performed with the isolate, carissone exhibited activity mostly against the Gram negative *B. subtilis* and *E. coli* (MIC 5mg/mL); Gram positive strains, however, were inhibited to a lesser extent. Two more compounds were isolated from the root's DCM extract, but conclusive structural elucidation and individual antibacterial studies could not be undertaken with those within the time frame of this project, particularly as only very small quantities were obtained in a pure form.

A MeOH extract of the root's wood was also studied with the aim to isolate and identify antibacterial compounds. After a series of chromatographic experiments the lignan carinol was isolated from the moderately polar fractions of the MeOH extract. Its IR and ^{1}H NMR characteristics and its R_f value in chloroform: EtOAc (1:4) compare well with data reported in the literature for this compound[28].

Carinol was also tested for its antibacterial properties and was shown to be active against *E. coli, S. aurues* and *B. substilis* (MIC 1.25 mg/mL), but did not *P. aeruiginosa*.

In summary, from the isolated compounds, the volatile principle 2'-hydroxy acetopheneone showed the highest activity against the four bacterial strains included in the antibacterial assays of this work, followed by the lignan carinol and the

eudesmane sesquitepene carissone. These findings support many of *C. lanceolata* root's traditional uses, which appear to be mainly attributable to an antimicrobial activity of the plant. Thus this project confirms, to an extent, its traditional use by many indigenous Australians communities of Northern Australia as a topical antibacterial herbal remedy.

Micro broth dilution and in association with this, the use of resazurine as an indicator was also demonstrated to be a very effective technique for antibacterial studies of crude plant extracts and isolated compounds.

Future studies on the root material should be focused on the two compounds, which were isolated in this work but could not be structurally identified nor tested for their anti bacterial properties. It would also be of interest to concentrate more strongly on the plant's leaves and twigs, which have not yet been fully explored for their chemical constituents and antibacterial activity.

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Appendices

Appendix I – Spectroscopic Data of Isolated Compounds

Figure 23: ^{1}H NMR Spectrum of 2′-Hydroxy acetophenone [#1.1]

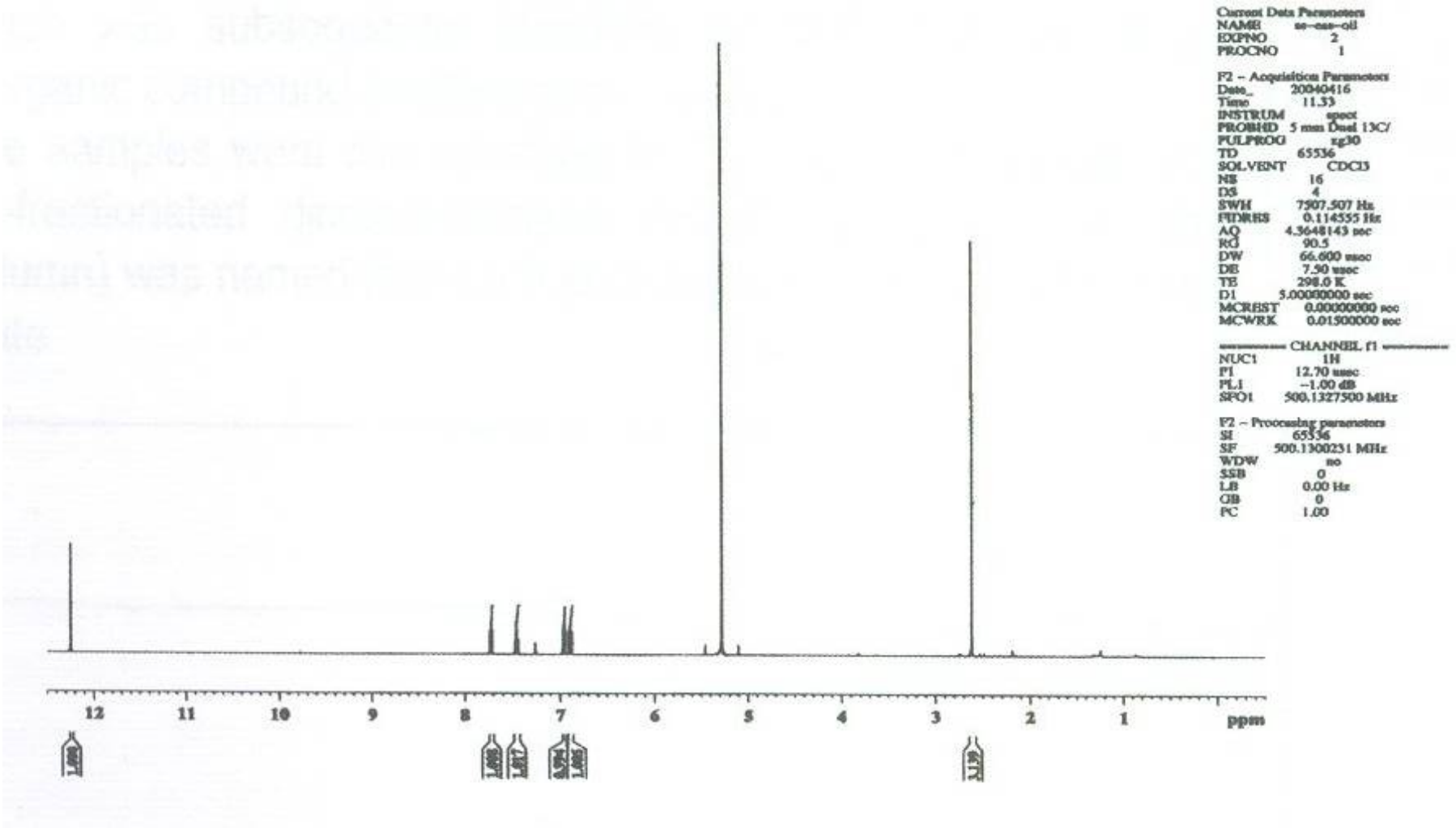


Figure 25: Mass Spectrum of 2′-Hydroxy acetophenone [#1.1]

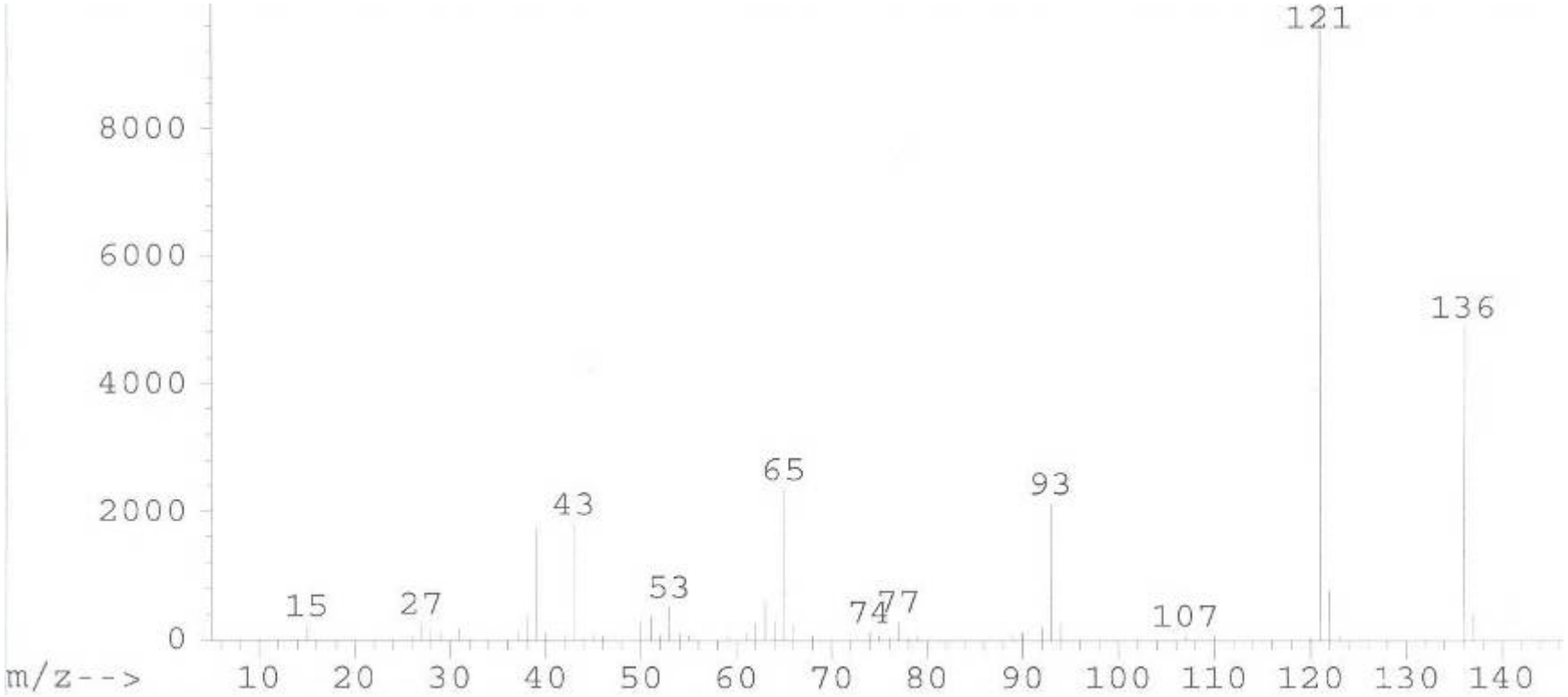


Figure 25: ^{1}H NMR Spectrum of Carissone [#4.2.2.1]

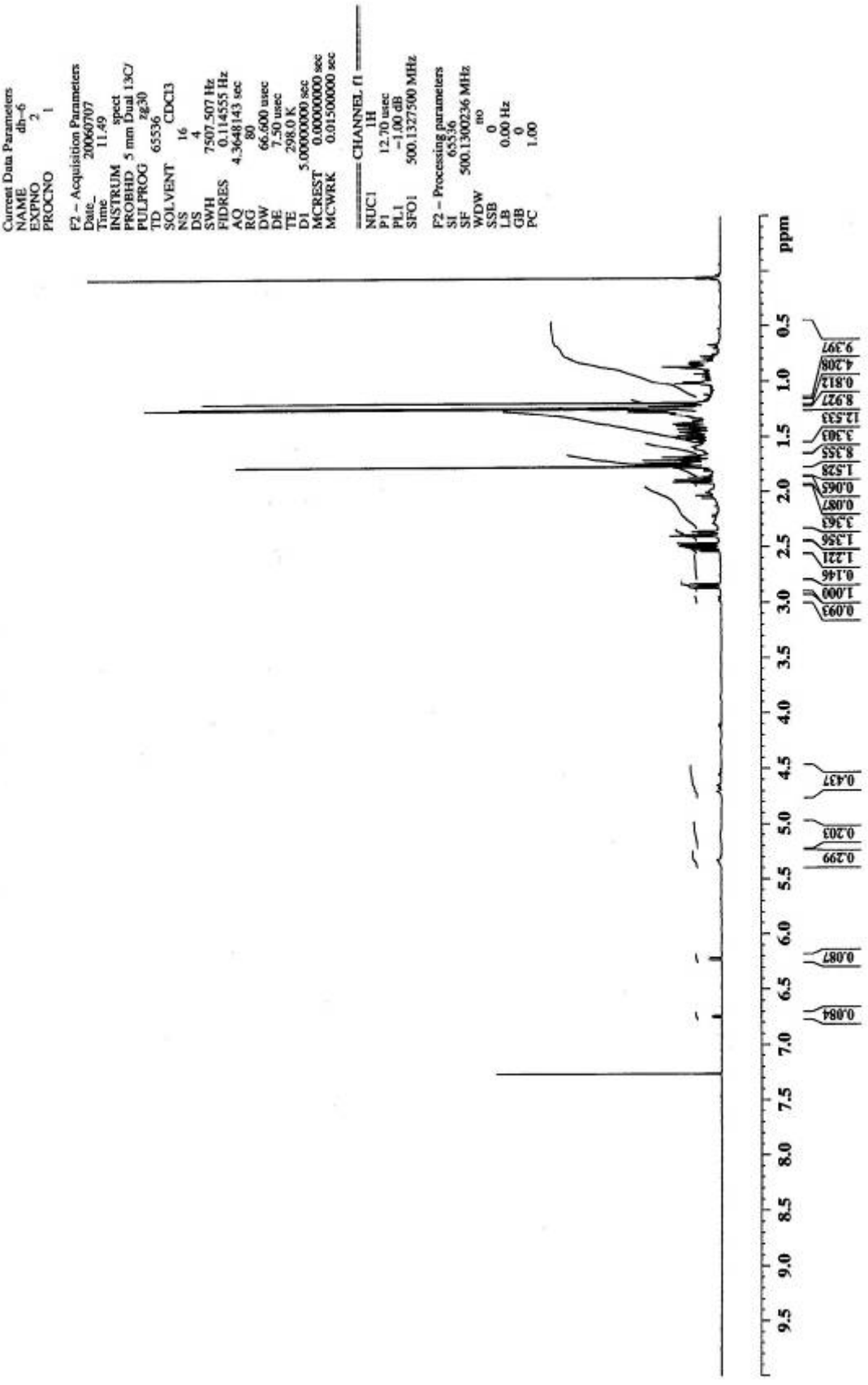


Figure 26: Expansion of ^{1}H NMR Spectrum of Carissone[#4.2.2.1] (2.75-2.15 ppm)

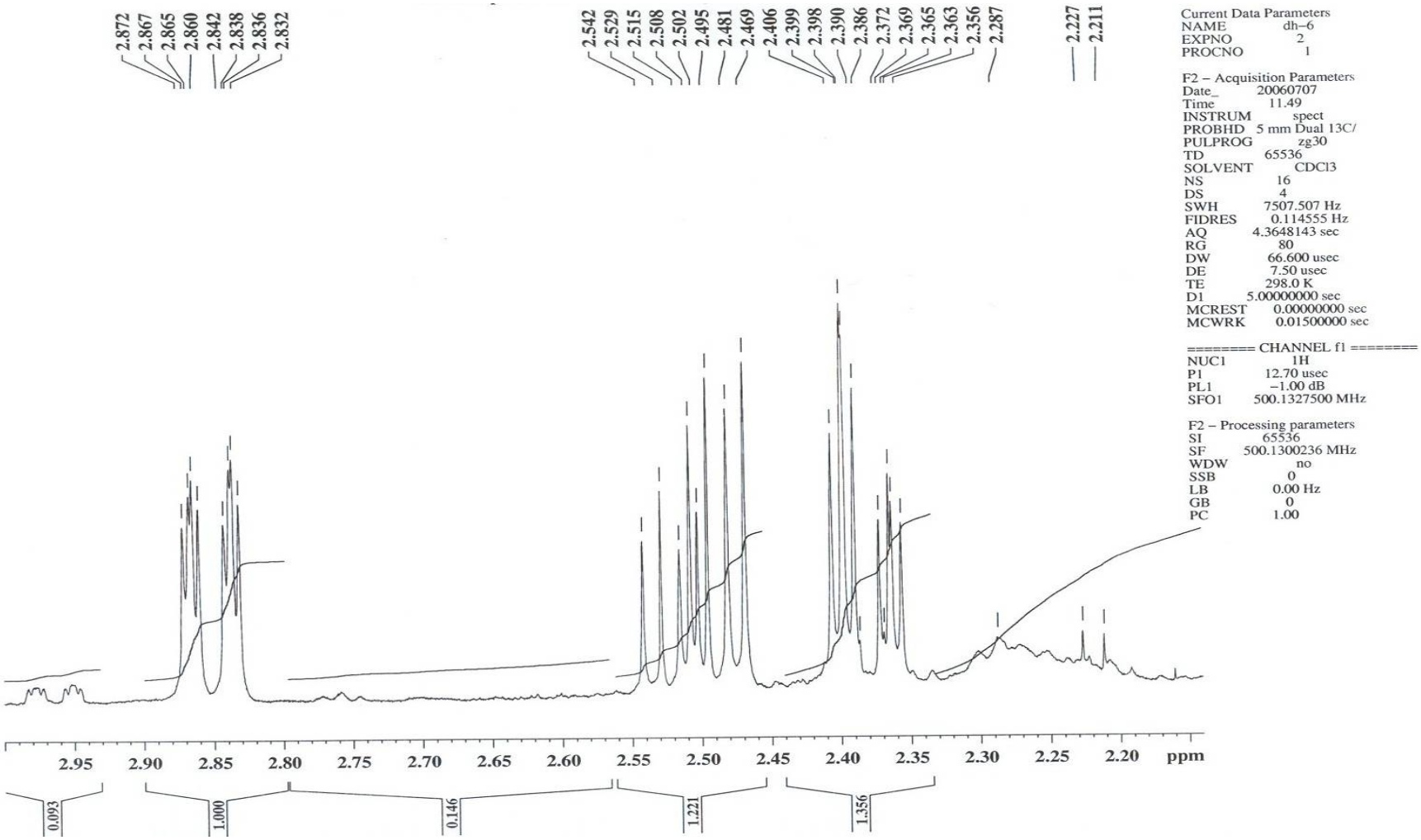


Figure 27: Expansion of ^{1}H NMR of Spectrum of Carissone [#4.2.2.1] (1.85-1.60 ppm)

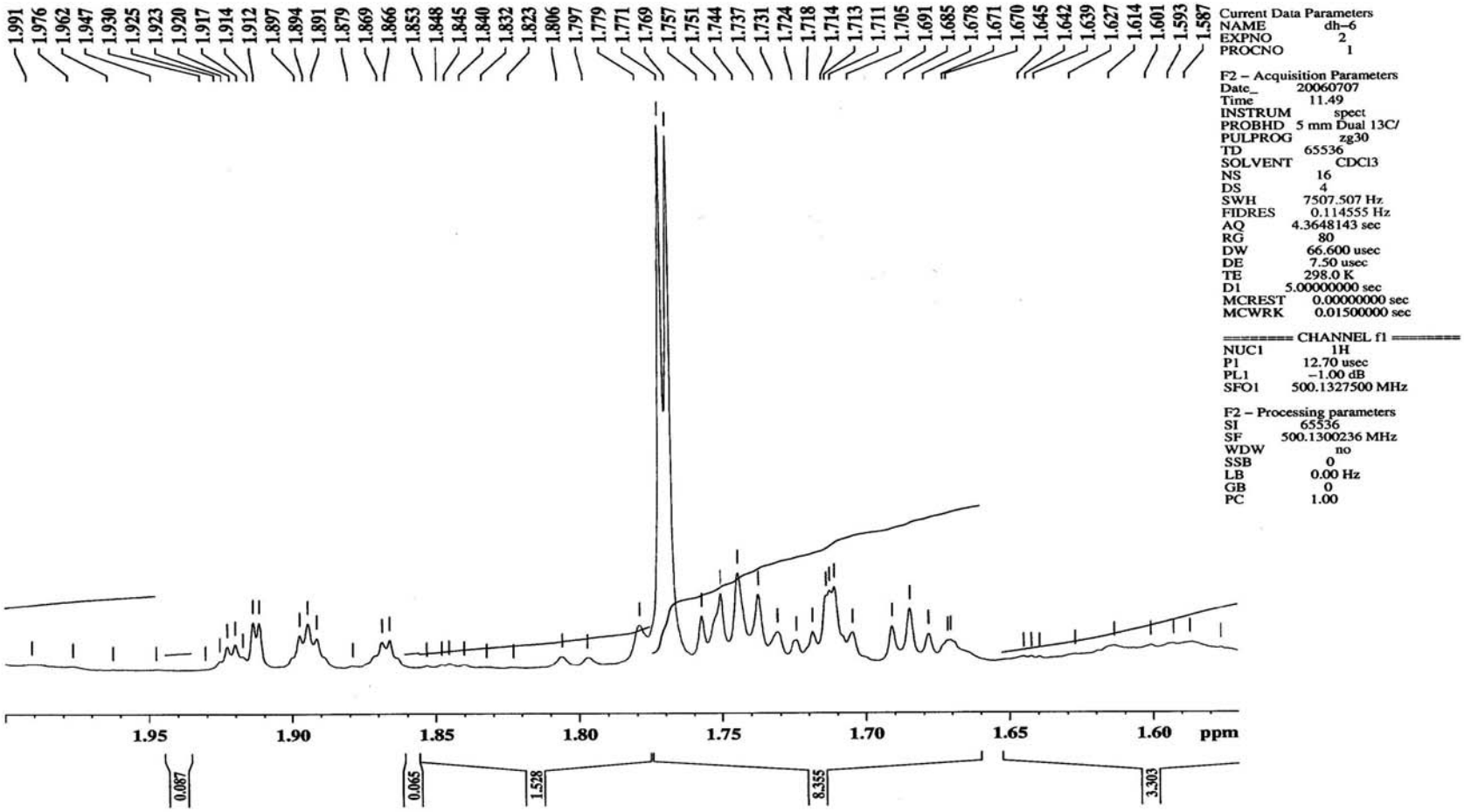
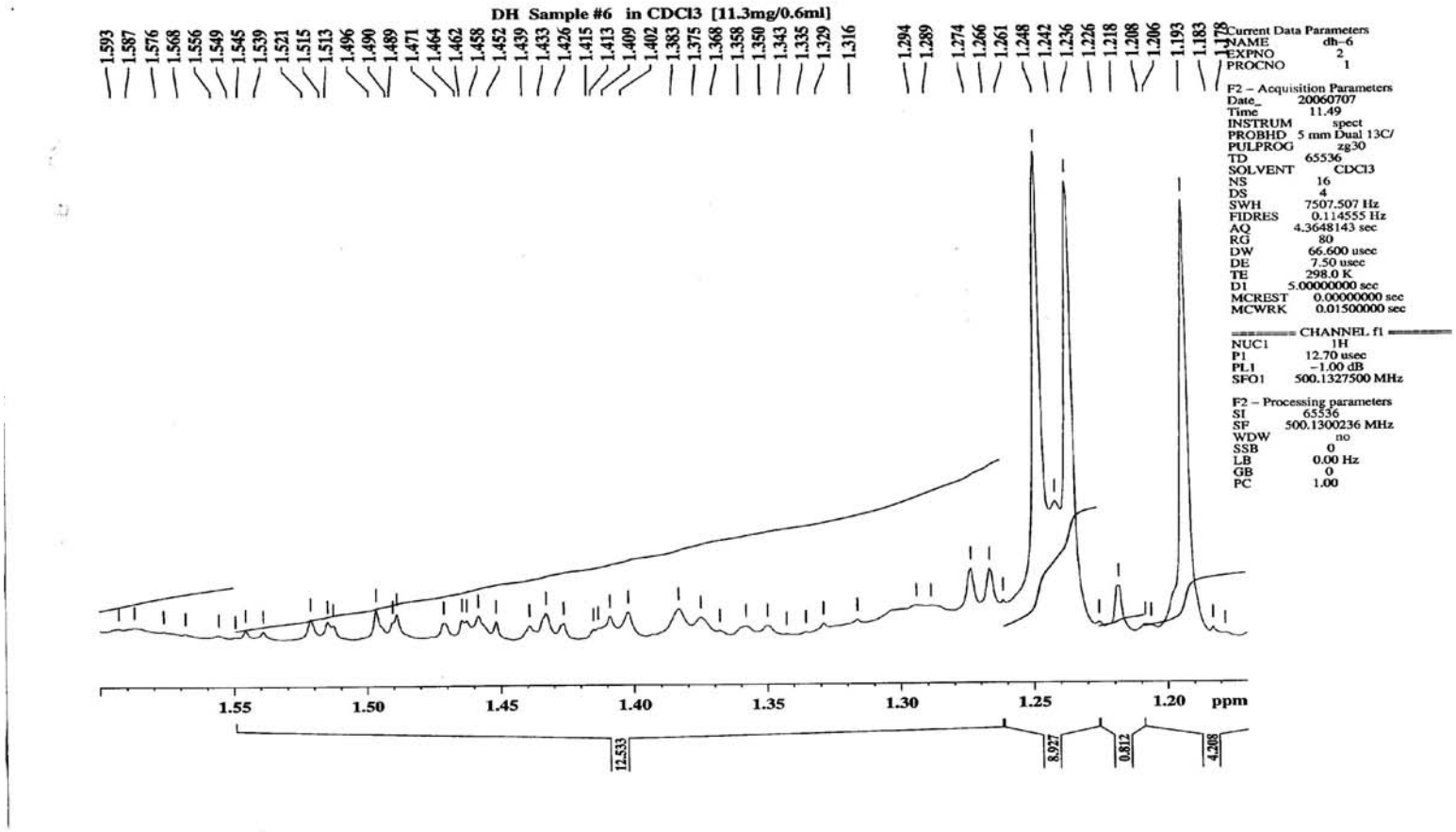


Figure 28: Expansion of ^{1}H NMR of Spectrum of Carissone [#4.2.2.1] (1.55-1.20 ppm)



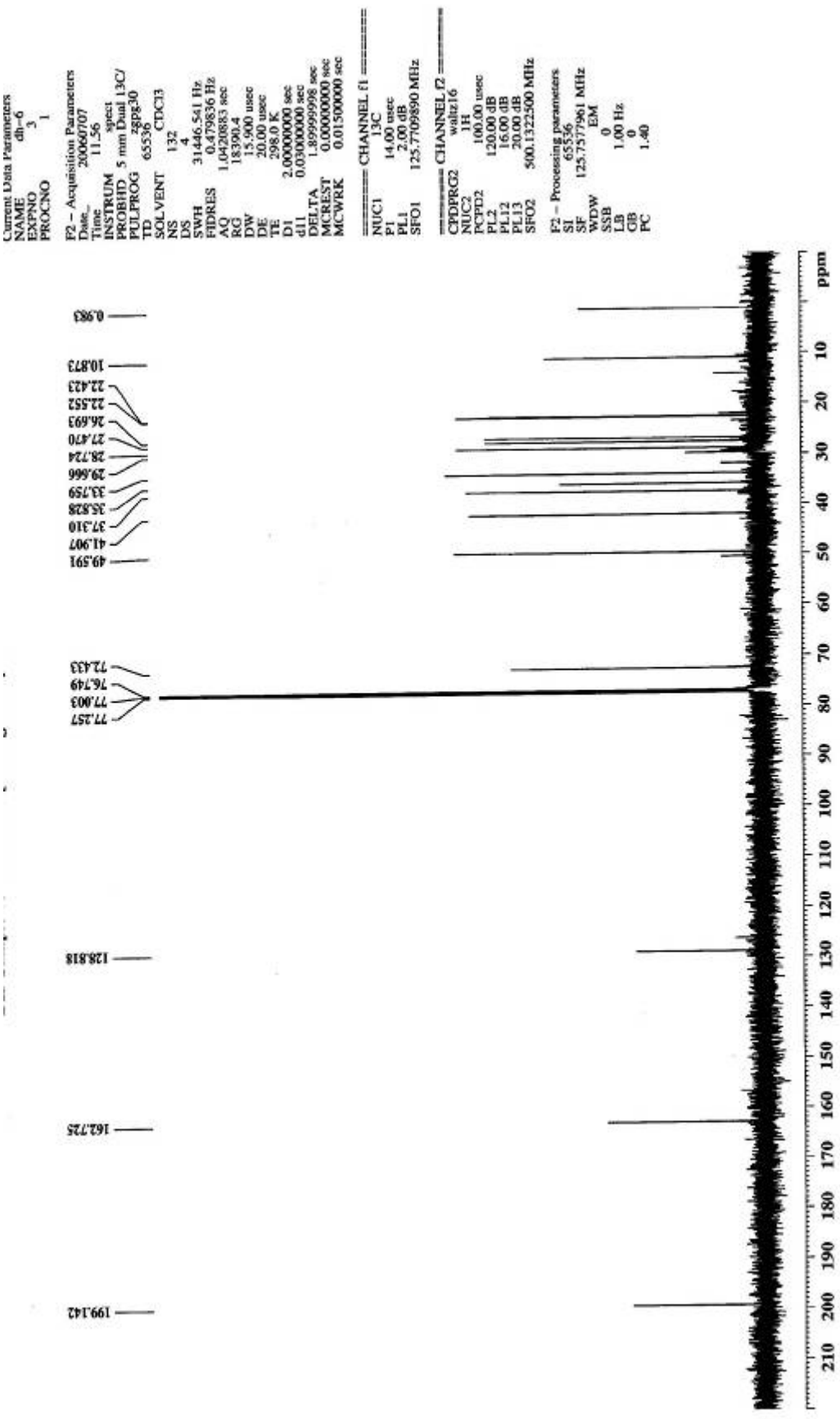


Figure 29: ^{13}C NMR Spectrum of Carissone [#4.2.2.1]

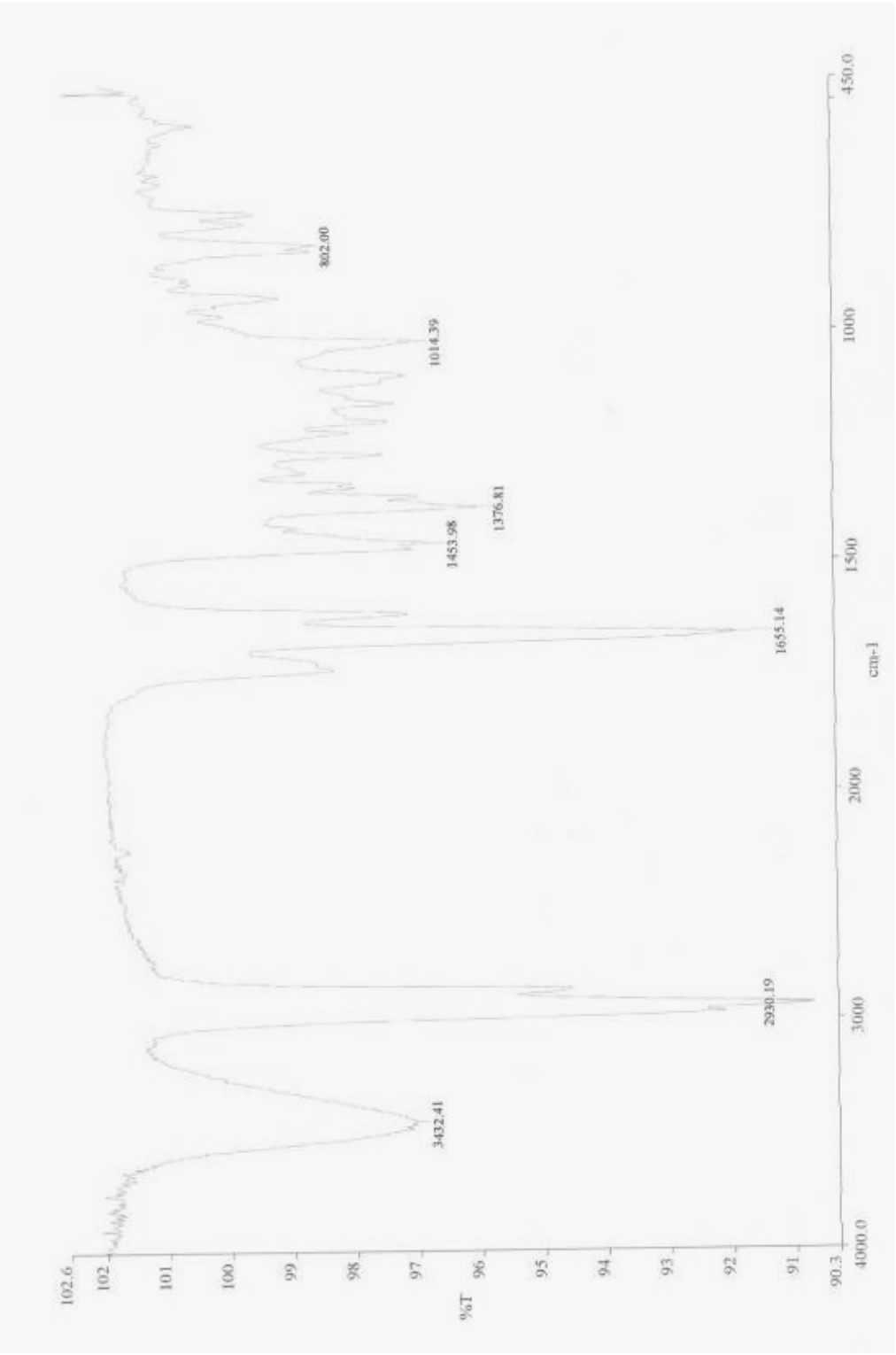


Figure 30: Infrared Spectrum of Carissone [#4.2.2.1]

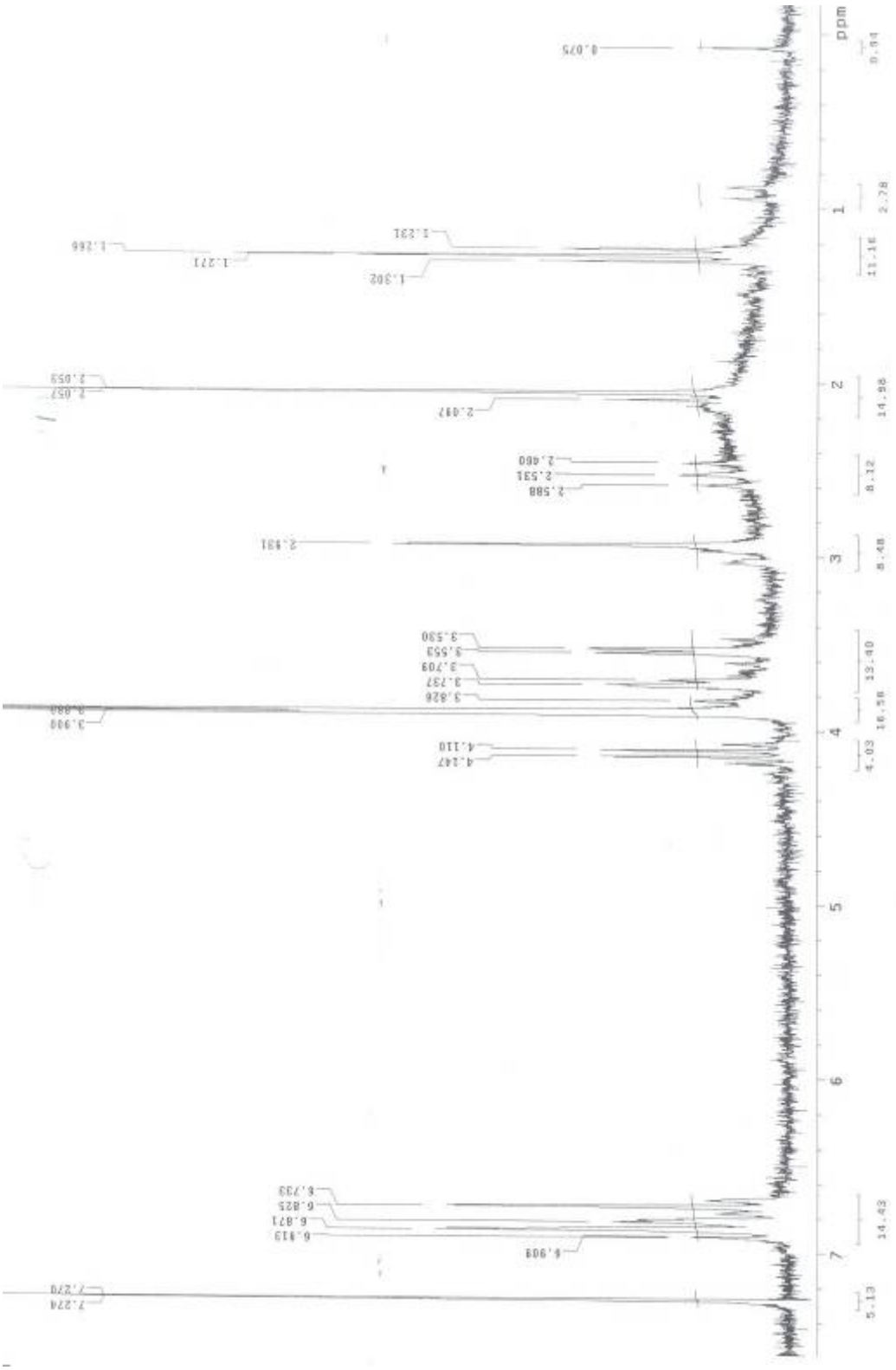
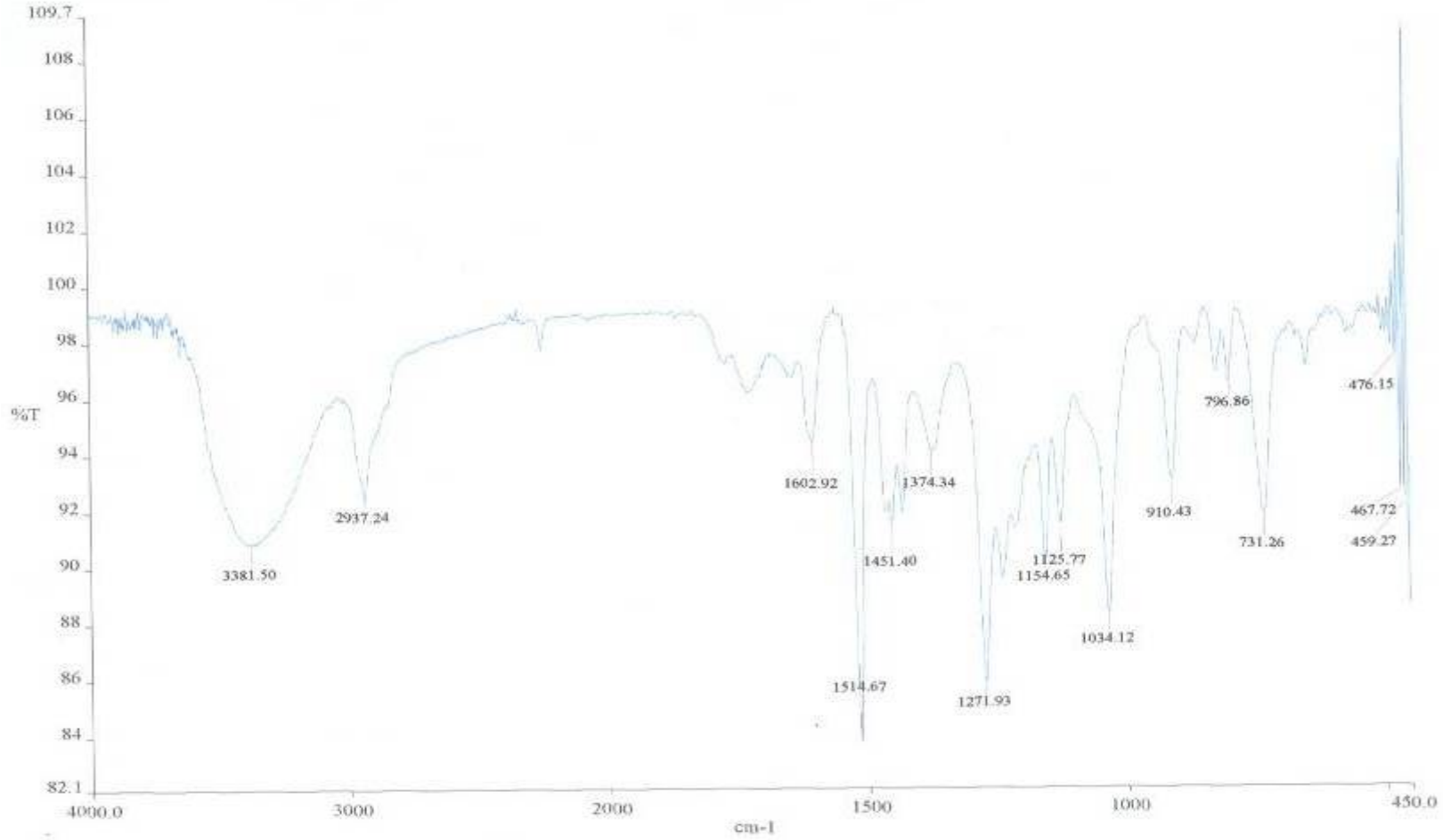


Figure 31: ^{1}H NMR Spectrum of Carinol [#8.1.2.1.2.2]

Figure 32: Infra-Red Spectrum of Carinol [#8.1.2.1.2.2]



Appendix II – Reagents

1. **Dragendroff's Reagent**

   Preparation:[73]

   Mix 2g of bismuth subnitrate, 25mL of acetic acid and 100 mL of water (a). Dissolve 40g of potassium iodide in 100mL of water (b). Mix 10 mL of (a) and 10 mL of (b), 20 mL of acetic acid and 100 mL of water.

2. Mayer's Reagent

   Preparation: [73]

   Dissolve 1.36g of mercuric chloride in 60 mL of water. Also prepare a solution of 5g potassium iodide in 20 mL of water. Mix both solutions and make up to 100 mL with water.

3. Anisaldehyde Spray Reagent

   Preparation:[73]

   Dissolve 0.5 mL of anisaldehyde in 10 ml of glacial acetic acid, add 85 mL of MeOH and 5 mL of sulphuric acid.

4. Ferric chloride (10%) reagent

   Preparation:[72]

   Iron (III) chloride is dissolved in water to obtain a 10% w/v concentration.

5. Ethanolic ferric chloride (5%) solution

   Preapration: [72]

   Dissolve Iron (III) chloride in ethanol (95%v/v) to obtain a 5% w/v concentration.

Appendix III – Method Development for Anti-Microbial Assay

In a preliminary project to this study, three different anti-microbial bioassays (disc diffusion, well diffusion and micro-broth dilution) were conducted and their results compared using various extracts of *Tinospora smilacina,* another important traditional Aboriginal medicinal plant. As the latter, particularly in combination with resazurine as indicator was found to be the most convenient and reliable assay, it was adopted as the method of choice for this work.

Tinospora smilacina, which is the Australian member of the genus Tinospora, is also known as "Snake Vine" and used in indigenous medicine by various Aboriginal communities in Northern and Western Australia. A literature survey revealed that little scientific work has to date been undertaken with this particular species, which is in contrast to the well researched Asian members of the family, *T. cordifolia* and *T. sinensis*. In this study methanol and hexane extracts of *T. smilacina* as well as selected extract fractions separated by column chromatography were subjected to anti-microbial activity assays using three different methods, namely disc diffusion, well diffusion and micro-broth dilution. One aim was to compare the results obtained with the three methodologies and, based on this, assess their suitability for bioassay guided fractionations. Out of the three methods trailed micro-broth dilution was found to be the most convenient and reliable. Five fractions isolated by column chromatography from the methanol extract of the plant showed promising antimicrobial activity, particularly against *S. aureus*, and should be investigated further in a follow up study.

Findings of the above preliminary project were presented at the following research symposia:

1. Western Australian Biomedical Research Institute (WABRI), Research Symposium (Perth, Australia, 29th April 2005).
2. Australian Society for Medical Research (ASMR), Research Week Symposium (Perth, Australia, 7th June 2005).
3. Australasian Pharmaceutical Science Association (APSA-ASCEPT) Annual Scientific Meeting (Melbourne, Australia, 5th December 2005).

Appendix IV – Presentations

Antibacterial activity of *Carissa lanceolata* R.Br. Root. Dhanushka S Hettiarachchi, Susanne Schorr & Connie Locher. School of Pharmacy, Curtin University of Technology, Western Australia 6152

Carissa lanceolata (Conkerberry) is a perennial woody shrub used in traditional medicine by indigenous communities in Western Australia, the Northern Territory and Queensland, for various medical conditions such as, toothache, respiratory infections, cleaning of sores, which all strongly indicate an antibacterial activity. A literature review revealed that the wood of this plant possesses significant antibacterial activity, which was found to be related to the presence of eudesmane type sesquiterpenes.

To expand on these findings in the present study the volatile and non-volatile constituents of the root of the plant were investigated for their antibacterial activity. A volatile oil was isolated from the root bark, both by solid phase extraction and steam distillation, which was found to constitute mainly 2-hydroxyacetophenone as identified by GC/MS and NMR analyses. MeOH and DCM extracts of the root bark and root wood as well as the phenolic 2-hydroxyacetophenone itself showed a considerable antimicrobial activity when assayed. A micro-broth dilution assay was performed on 96-well plates using resazurin as indicator for the microbial growth, against *Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis* and *staphylococcus aureus.* These extracts were fractionated by column chromatography and further separated by preparative TLC in an attempt to identify their antimicrobial constituents.

Presented to the following research symposia:

1. Australian Society for Medical Research (ASMR), Research Week Symposium (Perth, Australia, 9th June 2006).
2. 6th International & 16th National Chemistry Conference, Pakistan (Multan, Pakistan, 7th April, 2006).
3. Australasian Pharmaceutical Science Association (APSA-ASCEPT) Annual Scientific Meeting (Melbourne, Australia, 5th December 2005).